

**The analysis of *Metarhizium robertsii* potential as endophytic plant root coloniser, plant growth enhancer and antagonist to bean root pathogen**

***Fusarium solani* f. sp. *phaseolis*.**

By

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## 1. Abstract

The soil-inhabiting insect-pathogenic fungus *Metarhizium robertsii* also colonizes plant roots endophytically, thus showing potential as a plant symbiont. *M. robertsii* is not randomly distributed in soils but preferentially associates with the plant rhizosphere when applied in agricultural settings. Root surface and endophytic colonization of switchgrass (*Panicum virgatum*) and haricot beans (*Phaseolus vulgaris*) by *M. robertsii* were examined after inoculation with fungal conidia. Light and confocal microscopies were used to ascertain this rhizosphere association. Root lengths, root hair density and emergence of lateral roots were also measured. Initially, *M. robertsii* conidia adhered to, germinated on, and colonized, roots. Furthermore, plant roots treated with *Metarhizium* grew faster and the density of plant root hairs increased when compared with control plants. The onset of plant root hair proliferation was initiated before germination of *M. robertsii* on the root (within 1-2 days). Plants inoculated with *M. robertsii*  $\Delta$ MAD2 (plant adhesin gene) took significantly longer to show root hair proliferation than the wild type. Cell free extracts of *M. robertsii* did not stimulate root hair proliferation. Longer term (60 days) associations showed that *M. robertsii* endophytically colonized individual cortical cells within bean roots. *Metarhizium* appeared as an amorphous mycelial aggregate within root cortical cells as well as between the intercellular spaces with no apparent damage to the plant. These results suggested that not only is *M. robertsii* rhizosphere competent but displays a beneficial endophytic association with plant roots that results in the proliferation of root hairs.

The biocontrol of bean (*Phaseolus vulgaris*) root rot fungus *Fusarium solani* f. sp. *phaseolis* by *Metarhizium robertsii* was investigated *in vitro* and *in vivo*. Dual cultures on

Petri dishes showed antagonism of *M. robertsii* against *F. solani*. A relative inhibition of ca. 60% of *F. solani* growth was observed in these assays. Cell free culture filtrates of *M. robertsii* inhibited the germination of *F. solani* conidia by 83% and the inhibitory metabolite was heat stable. Beans plants colonized by *M. robertsii* then exposed to *F. solani* showed healthier plant profiles and lower disease indices compared to plants not colonized by *M. robertsii*. These results suggested that the insect pathogenic/endophytic fungus *M. robertsii* could also be utilized as a biocontrol agent against certain plant pathogens occurring in the rhizosphere.

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## 6. Introduction

One of the major constraints to increased agricultural production is the losses caused by insects, plant diseases and weeds. About 40% of the potential production is lost (Thacker, 2002) and losses remain relatively constant even after marked increase in pesticide use (Oerke, 2006). Therefore, development of agricultural practices which reduce adverse effects on the environment and result in products which are safe for human consumption is an emerging challenge in modern agricultural practices.

Entomopathogenic, or insect-pathogenic, fungi have been an important tool for insect pest control. Tremendous efforts are employed to find naturally occurring fungal pathogens capable of controlling pest insects. While this approach selects for strains pathogenic to target insects, it does not take into account the role of these fungi in their natural habitats. Thus, not all the potentials of these fungi in the environment are realised (Hajeck et al., 2007).

*Metarhizium*, an entomopathogenic fungus, is used globally as a biological control agent for many insect pests. It is also the best studied entomopathogenic fungus at the molecular and biochemical level (St. Leger et al., 2011). It follows the main criteria of being a biocontrol agent: high insect specificity, low toxicity to other organisms, low environmental impact (Miller et al., 1983), and less likeliness for the development of resistance from insects due to its multiple infection development processes (He and Xia, 2009). *Metarhizium* species are found in many parts of the world and have the ability to infect a wide range of insect hosts (Bidochka and Small, 2003; Samson et al., 1988). The mechanisms used by *M. anisopliae* to infect host insects are relatively well understood (St. Leger et al., 1986a). Under appropriate conditions, conidia of the fungus adhere by non-specific hydrophobic mechanisms to the insect surface (Boucias et al.,

1988). Once attachment is achieved, the conidia germinate and give rise to hyphal penetration of the insect cuticle (St. Leger et al., 1986b, 1988, 1991).

The infective propagules of this insect-pathogenic fungus, conidia, play a very important role in pathogenicity. In many countries, the production and harvesting of conidia of *Metarhizium* species have been commercially developed as an alternative to chemical pesticides (Goettel et al., 1995; Schulze et al., 2001). However, there are limitations to using *Metarhizium* species as biological control agents even though they are known to be environmentally safe (Roberts and Hajek, 1991; Milner, 2000; Milner and Hunter, 2001). Researchers have found that it is difficult to mass produce conidia since there can be a natural decrease in production of these infective propagules during successive subculturing. Furthermore, some conidia can be ineffective and unstable under field conditions (St. Leger and Bidochka, 1996; Kamp and Bidochka, 2002).

*Metarhizium* has been considered as a saprophytic organism having both soil-dwelling and pathogenic life stages (Roberts and Humber, 1981). However, in recent studies certain *Metarhizium* spp. may also be linked to the root rhizosphere (the layer of soil influenced by root metabolism) (St. Leger et al., 2011). *M. robertsii* when uniformly inoculated in a field was found to be preferentially colonizing root rhizosphere over bulk soil. Differential expression of two adhesion genes, *MAD1* and *MAD2* (Wang and St. Leger, 2007) was found in *Metarhizium* exposed root exudates. *MAD1* is related to adhesion of *Metarhizium* to insect cuticles while *MAD2* has been linked to plant adhesion. Rhizosphere competence of *Metarhizium* could play an important role in utilization of the fungus for insect control.

The objective of this study was to examine the associations of *M. robertsii* with two economically important plants, switchgrass and haricot beans. *M. robertsii* was found to colonize plant roots endophytically and promoted plant root development. This shows the potential of *M. robertsii* as a plant symbiont. The ability of *M. robertsii* to control the bean root-rot pathogenic fungus *Fusarium solani* f. sp. *phaseolis* was also studied. *M. robertsii* can no longer be simply defined as insect-pathogenic. *Metarhizium robertsii* could be used in multiple roles, from protecting plants from pests to promoting plant growth and protection against plant pathogens.

The present study focuses on the soil/root interface as a site where plants, the biocontrol fungus *M. robertsii*, and pathogens interact. There were two parts to this study, each with different objectives. Firstly, associations of *Metarhizium robertsii* ARSEF 2575 with plant roots were observed microscopically. Plant roots were also observed macroscopically for root lengths, lateral root emergence, and root hair density. Secondly, *M. robertsii* was evaluated for antagonistic activity against the bean root-rot pathogen *Fusarium solani* f. sp. *phaseolis*. *M. robertsii* was shown to suppress the pathogenic fungi *F. solani* f. sp. *phaseolis* in both plate assays as well as plant assays.

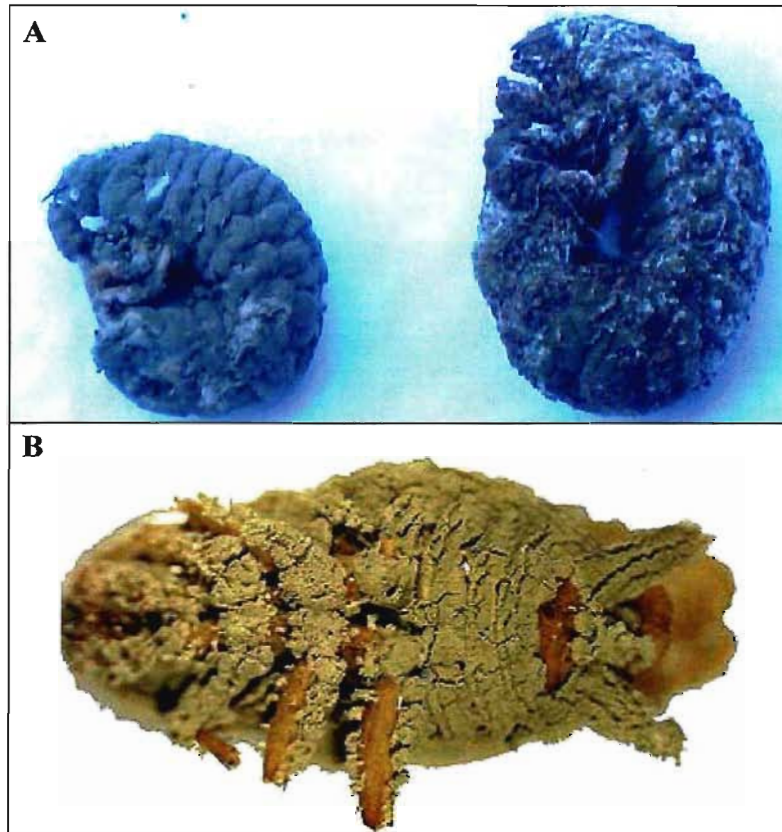
## 7. Literature Review

### 7.1. *Metarhizium* as a Biocontrol Agent

The genus *Metarhizium* is the most intensively investigated mycoinsecticide in the Clavicipitaceae family, which has been used for years on a large scale in different countries (Kalia and Mudhar 2011). The fungus is known to cause green muscardine disease in insects because of its green spores (Fig. 1). The entomopathogenic fungus is effective against various insect species (Lomer et al., 2001; Milner and Pereire, 2000; Hunter et al., 2001; Maniania et al., 2003; Shah and Pell, 2003). *M. anisopliae* is commercially available for the control of pests on pasture turf, white grubs, mole crickets, caterpillars and termites (St. Leger, 2007).

*Metarhizium* has been scientifically classified in the kingdom Fungi, phylum Ascomycota, class Sordariomycetes, order Hypocreales and family Clavicipitaceae. These fungi are able to flourish in soil or survive there in a dormant state waiting for a susceptible host. *Metarhizium anisopliae*, formerly known as *Entomophthora anisopliae*, is an important and the most studied member of the genus and is recoverable from soil world-wide (St. Leger et al., 2011). The first use of *M. anisopliae* as a microbial agent against insects was in 1879, when Elie Metchnikoff used it in experimental tests to control the wheat grain beetle *Anisoplia austriaca*. *M. anisopliae* is a mitosporic fungus with asexual reproduction, and conidia (asexual spores) are the infecting agents.

*M. anisopliae* was found to occur more frequently in agricultural habitats than in forests (Bidochka et al. 1998). Genetic groups of *M. anisopliae* are linked to habitat type rather than insect host, suggesting that selection for survival in the soil is more important in shaping the population genetics of *M. anisopliae* than is selection for pathogenicity (Bidochka, 2001).

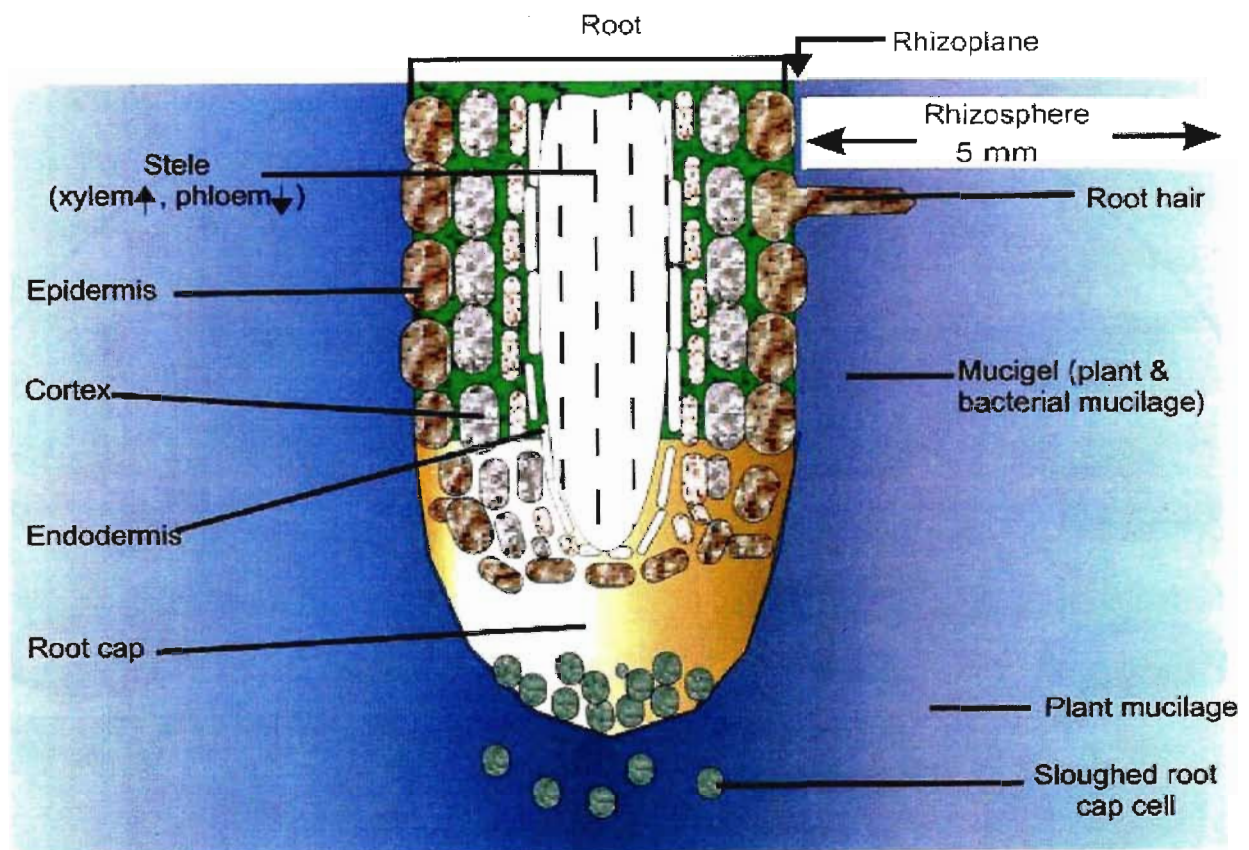


**Figure 1.** *Metarhizium* infected dead insects. (A) *Metarhizium* infected white grub (Yubak Dhoj, 2006). (B) *Metarhizium anisopliae* infected cockroach. (Source: CSIRO Entomology; 13 <http://www.scienceimage.csiro.au/index.cfm?event=site.image.detail&id=2765>)

Bidochka (2001) revealed two non-recombining lineages of *M. anisopliae* var. *anisopliae* in southern Ontario, Canada. Among them, one lineage typically occurred in agricultural soils while the other was more common in forest soils. Recently, in a phylogenetic study done by Bischoff et al. (2009), *M. anisopliae* var. *anisopliae* has been reclassified as *M. robertsii*. A large population of insect hosts could contribute to *Metarhizium* soil populations. However, it is found that populations as large as those characterized for *M. robertsii* are normally the result of organic substrates in rhizospheres of the upper layers of soil (St. Leger, 2007). Given that rooting density is high in grasses and cereal crops (with less than 3mm spaces between roots) (Barley, 1970), it is now suggested that *Metarhizium* communities must be living in overlapping rhizospheres. *M. robertsii*, therefore, has two distinct lifestyles; as an insect pathogen and as a soil-dwelling saprophyte associated with plant roots at the rhizosphere (Hu and St. Leger, 2002).

## **7.2. Rhizosphere and Rhizosphere Competence**

Hiltner, in 1904, described the “rhizosphere” as a zone of unique and dynamic interactions between plant roots and soil micro-organisms (Curl and Truelove, 1986) (Fig. 2; Raina Maier, 2000). This specialized region is characterized by enhanced microbial biomass and activity. Microbiota (bacteria, fungi, and algae) and micro- and mesofauna (protozoa, nematodes, mites, and insects) constitute the rhizosphere community (Curl and Truelove, 1986). Rovira, in a review in Microbiology (1965), redefined the rhizosphere as a poorly defined zone of soil with a microbiological gradient. In this gradient, the maximum changes to microflora occur in the soil adjacent to the root and decline with distance away from the roots. The complex interaction between plant roots and micro-organisms is influenced by several factors. Such factors affecting



**Figure 2.** Representation of plant rhizosphere as given by Raina Maier *et al*, 2000, Environmental Microbiology.

interactions between plant roots and micro-organisms include root exudates (most important), plant species, age of the plant, foliar sprays, soil conditions and environmental conditions.

Important components of the rhizosphere are the roots themselves as they provide plants with anchorage and the means to acquire water, nutrients and other growth substances from soil. In addition, roots provide rhizosphere micro-organisms with highly favourable growth conditions in the form of structure for microbial colonization and inputs of photosynthate to the rhizosphere (Walton et al. 1994). Some of the most complex interactions (chemical, physical and biological) occur between roots and the rhizosphere community. These interactions, as mentioned by Bais et al. (2006), could involve root-root, root-insect, and root-microbe interactions. Thus, the rhizosphere is a highly dynamic front for interactions between roots and pathogenic and beneficial soil microbes, invertebrates, and the root systems of competitors (Hirsch et al. 2003).

Plant root exudates determine the outcome of all the interactions taking place in the rhizosphere (Bais et al., 2006). Root exudates are continuously secreted compounds from plant roots (Bais et al., 2001; Gleba et al., 1999). The compounds exuded by plants include ions, mucilage, and a diverse array of carbon-containing primary and secondary metabolites (Bertin et al., 2003; Bais et al., 2006). Microbial activity in the rhizosphere is substantially increased with the presence of root exudates (Oger et al., 2004).

### **7.3. Plant Fungal Interactions**

Fungi are the second most prevalent group of microorganisms in soil and occasionally fungal biomass exceeds bacterial biomass. Interactions between plants and fungi could be beneficial, neutral or harmful. Pathogenic root-infecting fungi, saprophytic and mycorrhizal



fungi are among the three major types of soil fungi (Walton et al., 1994). Plants benefit from these fungal associations through enhanced water and mineral uptake, increased resistance to pathogens, and tolerance to environmental stresses (Walton et al., 1994). Benefits are not limited to plants, and occasionally fungal partners also benefit from nutrients exuded by plants. Non-mutualistic fungi (e.g., *Fusarium* spp.) also occur in the rhizosphere and rhizoplane (root surface) (Walton et al., 1994). Microbial colonization of the rhizosphere is an important first step in pathogenesis by soil-borne micro-organisms and a crucial one in the application of beneficial micro-organisms (Bais et al., 2008).

### **Positive Interactions Mediated by Root Exudates**

Interactions between plants and microbes can positively influence plant growth through a variety of known mechanisms. Nodulation of legumes by rhizobia, mycorrhizal associations, endophytic associations, and plant growth promoting factors produced by bacteria or fungi are the most established mechanisms in these types of interactions. Microbe populations in the rhizosphere have influences on various physiological aspects of plant growth including root morphology, root-to-shoot weight ratio, uptake of nutrients like calcium, phosphorous and several other minerals (Singh et al., 2004; Bais et al., 2006), rate of development and onset of flowering, and crop yield (Rovira, 1965).

### **Interactions and Antagonisms in the Rhizosphere**

Interactions between various members of the rhizosphere are equally important for influencing the balance between these organisms as the effect of roots (Rovira, 1965). It has been shown that various mechanisms are involved in the rhizosphere which could increase the growth of a pathogen as well as causing many rhizosphere isolates to antagonise these pathogens.

Results obtained have shown that root exudates of peas susceptible to *F. oxysporum* stimulated the fungus in pure cultures, and caused many rhizosphere isolates to antagonize the pathogen. These results indicate two opposite mechanisms that are operating in the rhizosphere of susceptible pea plants (Buxton, 1957; Rovira, 1965).

With increasing evidence of beneficial and detrimental effects of rhizosphere microbial populations upon higher plants, studies are now focusing on modifying the rhizosphere to encourage the development of favourable micro-organisms and discourage adverse types (Rovira, 1965).

#### **7.4. Fungal Endophytes**

Petrini (1991), named all organisms which inhabit plant organs and can colonize internal plant tissues without causing apparent harm to the host as endophytes (Hyde and Soyong, 2008). Fungal endophytes were defined as fungi that colonize a plant without causing visible disease symptoms at any specific moment (Schulz and Boyle, 2005). Endophytes could be found colonizing any organ from leaves to roots, and could also be tissue specific (Tao et al., 2008). Additionally, they have been isolated from all types of plants ranging from large trees, palms, sea-grasses, and even lichens. Both ascomycetes and basidiomycetes could form endophytic association with plants.

Endophytes, depending on their roles, have been classified as clavicipitalean (grass-inhabiting) and non-clavicipitalean (generally non grass-inhabiting). Clavicipitalean endophytes are known to have various functions including enhancing the resistance of grasses to multiple stresses (Kuldau and Bacon, 2008). The non-grass-inhabiting endophytes are known to be involved in mutualism, decreased herbivory, increased drought resistance, increased disease

resistance and enhancement of plant growth (Hyde and Soyong, 2008; Frohlich et al., 2000; Sieber, 2007).

Green fluorescent protein (GFP) labelled organisms can be used to study the symbiosis between plant roots and microbes. This technique could be used to understand the localization of microbes on/in different parts of plants including roots. For example, green fluorescent protein-labelled *Rhizobium meliloti* cells have been used to visualize the early events of symbiosis with alfalfa (*Medicago sativa*) (Gage et al., 1996). This use of a GFP labelled organism allows monitoring and evaluation of the dynamic interactions with host in near real conditions without the need of using sterile conditions, which would otherwise get destroyed with fixation or staining.

The biological control (biocontrol) of insects, phytopathogens, and diseases is generally achieved by using specific microbial agents or introducing selected microorganisms into the system. In these cases, the isolation of an efficient biocontrol agent must be developed. For such, endophytes are currently employed. Certain endophytic fungi and bacteria have proven efficient against phytopathogens, suggesting antimicrobial production. Endophytic fungi are isolated from plants and are subjected to tests for antimicrobial activity, in order to investigate their potential for agricultural application. Studies have indicated that endophytes have potential for biological control due to their antagonistic effect against the fungus *Sclerotinia sclerotiorum* (Curl and Truelove, 1986). Some *Trichoderma* species which are rhizosphere competent (Ahmad and Baker, 1987) and used as biocontrol fungi against plant pathogenic fungi (Harman, 2000) have also been shown to be endophytic plant symbionts (Yedidia et al., 1999). *Piriformospora indica* and *Sebacina vermifera* also form endophytic associations with plants and have been reported to control *Gaeumannomyces graminis* var. *tritica* (causal agent of “take all” disease in wheat)

(Ghahfarokhi and Goltapeh, 2010). Fungal entomopathogens like *Beauveria bassiana* and *Lecanicillium* spp. have been reported to provide dual biological control of both insect pests and plant pathogens (Owenly et al., 2010; Kim et al., 2007). An array of bioactive metabolites is produced by *Beauveria* spp. which is used in direct inhibition of pests as well as pathogens. *B. bassiana* is also reported to have endophytic colonization of tomato and cotton seedlings and protect them against pathogenic *Rhizoctonia* spp. and *Pythium myriotylum* (Ownley et al., 2008).

Our understanding of the ecology of entomopathogens outside their host as well as our ability to effectively utilize entomopathogens as biological control agents has increased. For example, Lewis and colleagues (Bing and Lewis, 1991, 1992) observed that *Beauveria bassiana* grows endophytically within the green tissues of *Zea mays*. It was later demonstrated that endophytic isolates of *B. bassiana* were effective in managing populations of the European corn borer, *Ostrinia nubilalis* (Lewis et al., 2002) while being non-pathogenic to *Z. mays* (Lewis et al., 2001).

### **7.5. *Metarhizium* - Rhizosphere Interactions**

Hu and St. Leger (2002) demonstrated the ability of *Metarhizium* (*M. anisopliae* ARSEF 1080) to colonize the rhizosphere by using a GFP expressing *Metarhizium* strain. They showed that *Metarhizium* is not randomly distributed in the soil but exhibits preferential associations with the plant rhizosphere. *Metarhizium* was found to be more persistent in rhizosphere than in bulk soil (Hu and St. Leger, 2002), and had better biocontrol activity within it (Bruck, 2005). Fungal population increase in the rhizosphere is a well reported notion, but the 2002 study by Hu and St. Leger was the first report of an entomopathogenic fungal population increase in the rhizosphere. The populations of *M. anisopliae* decreased in bulk soil, thus

suggesting involvement of root exudates in the rhizosphere effect (Hu and St. Leger, 2002) or enhanced sporulation in the rhizosphere. Root-deposited photosynthate is considered an important source of readily available carbon for microbes in the rhizosphere (Butler et al., 2003). Positive response to root exudates by *M. anisopliae* was also demonstrated by Klingen et al. (2002), though there was no quantification of rhizosphere fungal population. Later, different isolates of *M. anisopliae* (F52, IP99, and IP285) were shown to be rhizosphere competent for *Picea. abies*, *Picea glauca* and *Taxus bacata* in soilless potting media (Bruck, 2005).

*M. robertsii* adheres to insects and plants using two different proteins, MAD1 (*Metarhizium* ADhesin-like protein 1) and MAD2. These were found to be differentially induced in insect hemolymph and plant root exudates respectively, and produced adhesive conidial surfaces (Wang and St. Leger, 2007). Expression of *MAD1* and *MAD2* in *Saccharomyces cerevisiae* allowed yeast cells to adhere to insect cuticle and to plant surfaces, respectively. *Metarhizium* is thus a rhizosphere competent fungus with even more potential for biocontrol (St. Leger, 2008; Bruck, 2010).

Important to the rhizospheric competency of *Metarhizium* is a novel type of oligosaccharide transporter, MRT (*Metarhizium* Raffinose Transporter), unique to ascomycete and basidiomycete filamentous fungi (Fang and St. Leger, 2010). Disruption of the *MRT* gene had no effect on virulence to insects, but rhizosphere competency of *M. robertsii* was greatly reduced. A recent study by Gao et al. (2011), using comparative transcriptomics, showed *Metarhizium* to share >16% gene identity with plant pathogens which leads into the idea of *Metarhizium* having evolved as fungi adapted to growing on plants.

A Finnish isolate (V245) of *M. anisopliae* was reported to penetrate some leaf surfaces after conidia germination (Inyang et al., 1999). The potential of *M. robertsii* Mr-2575 as a root endophyte was not observed or reported.

## **7.6. Plant Protection against Microbial Plant Pathogens**

Plants are multicellular autotrophic eukaryotes prone to diseases caused by various abiotic and biotic factors. Among other factors, diseases caused by infectious agents such as bacteria, fungi, viruses and viroids are an important branch for plant protection (Montesinos, 2000). By virtue of several unique mechanisms and pathways that emerge during plant-microbe interactions, microbes offer a dual role; as causative agents of a disease or as biocontrol agents (Kalia and Mudhar 2011).

In plant pathology, the term “biological control” refers to the decrease in inoculum or the disease producing activity of a pathogen which is accomplished through the use of one or more organisms, including the host plant (Baker 1987). Some level of biocontrol of plant pathogens can be found in all agricultural ecosystems displayed by noticeable reduction in disease symptoms as compared to ecosystems experiencing disease pressure.

Soil is a common substrate between plants and soil borne pathogens. Thus, soil borne pathogens cause mild to severe diseases in plants. Depending upon the severity of infection, yield losses could take place.

The microbial live cells introduced or inoculated in or onto the plant to curb/control fungal plant pathogens are called biofungicides or biocontrol fungi (BCF). These have the

ability to control numerous foliar, root, and fruit pathogens. BCFs can also enhance nutrient uptake in plants, and substantially increase nitrogen use efficiency in crops. Some species also have the ability to increase the photosynthetic efficiency in plants. These abilities add to the importance of BCFs.

*Trichoderma* spp. is a common inoculant used and supplied as a biofungicide. Most of the initial work on biocontrol of plant diseases by *Trichoderma* spp. revolved around the ability of these fungi to interact with soil pathogens. The mechanisms used by *Trichoderma* in doing so are mycoparasitism, production of antibiotics, and competition for nutrients in the rhizosphere (Chet 1987; Harman and Shores, 2007; Harman et al., 2004a; Vinale et al., 2008). Many *Trichoderma* strains have the capability to colonize plant roots of dicots and monocots (Harman and Shores, 2007). During this process, *Trichoderma* hyphae coil around the roots and penetrate the root cortex (Yedidia, 1999). The fungus then grows intercellularly in the root epidermis and cortex and induces the surrounding plant cells to deposit cell wall material and produce phenolic compounds, thus inducing systemic defenses against plant pathogens. *Piriformospora indica*, the model system for *Sebacinales* fungi, also colonise roots. *P. indica* induces and modulates plant defences in roots by the production of gibberellins (Schafer et al., 2009). *Streptomyces griseoviridis* K61 has also been reported to show potent biofungicide ability to curb damping off of cauliflower caused by *Alternaria* (Kalia and Mudhar, 2011).

Fungi are known to secrete a wide array of compounds with biological activity against other organisms, which are mostly products of secondary metabolism. Depending on the ecological niche of the fungus, these metabolites could serve different functions. Some metabolites may be antibiotics to protect the BCF against antagonistic micro-organisms, or may prevent growth of saprophytic microbes on the host after it is killed, and thus improve the

survival of the BCF. *Metarhizium anisopliae* is reported to produce more than 27 types of destruxins, swainsonine and cytochalasin C which are known to be biologically active against a wide range of insect pests. *Beauveria bassiana* is another entomopathogen that produces secondary metabolites which also target insects, namely: bassianin, beauvericin, bassianolide, beauverolides, and tenellin. Similarly, *Trichoderma* is known to target fungi with secondary metabolites like harzianic acid, alamethicins, tricholin, peptaibols, antibiotics and many other compounds (Alain et al., 2001).

Entomopathogenic fungi such as *Metarhizium anisopliae* (St Leger and Bidochka, 1996), the nematophagous biocontrol agents *Paecilomyces lilacinus* and *Arthrobotrys oligospora* (Ahman et al., 2002; Bonants et al., 1995), as well as mycoparasitic fungi belonging to the genus *Trichoderma* (Geremia et al., 1993), have been found to secrete proteases in the presence of their hosts. The proteases are believed to facilitate the penetration into the host tissue by degrading the protein linkages in the host's external layers (the insect cuticle, nematode eggshell and fungal cell wall, respectively) and/or for the utilization of the host proteins for nutrition (Pozo et al., 2003).

*Metarhizium anisopliae* is now known to be a rhizosphere competent fungus, and is also known to produce secondary metabolites that target insect populations, and to secrete proteases. Surely these important characteristics of *Metarhizium* could be used by the fungus in the presence of plant pathogenic fungi as well.



## 7.7. Growth Promotion and BCF

BCF can also enhance plant growth (Shoresh et al., 2010). *Trichoderma* and *Sebacinales* species inoculations induce root and shoot growth ( Barazani et al., 2005; Harman, 2000; Harman et al., 2008; Harman *et al.*, 2004a; Harman *et al.*, 2004b; Peskan-Berghofer et al., 2004 ; Rai et al., 2001). *Trichoderma* application has led to an increase in dry matter content, starch, total and soluble sugars, and a reduction in sugar content in leaves of different plants (Adams and De-Lij, 2007; Lamba et al., 2008; Shoresh and Harman, 2008). *P. indica*, can promote adventitious root formation in plant cuttings obtained for vegetative propagation (Druege et al., 2007; Singh et al., 2000). More importantly, the effect of BCF on plant growth has a long duration and lasts for entire life of annual plants (Barazani et al., 2005; Harman, 2000; Harman et al., 2004a; Waller et al., 2005). Other non-pathogenic root colonizing fungi also have similar abilities (Lindsey and Baker, 1967).

Application of BCF could have direct and indirect effects on the plant growth promotion. Among the direct effects, metabolite production in the form of growth hormones and enhanced transfer of minerals to rhizosphere by the BCF enhances plant growth. Sometimes just the physical presence of a mycelial mass in the rhizosphere of plants would serve as bridge, enhancing nutrient uptake by plants. Indirectly, the BCF is responsible for induction of plant defence mechanisms. Also, an important mechanism in plant growth promotion by BCF is by counteracting deleterious root-associated microflora (Shoresh et al., 2010). Many *Trichoderma* spp. are known to use all of these mechanisms to promote plant growth (Verma et al., 2007).

Previous studies on *Metarhizium* as plant growth promoting fungus show *M. anisopliae* strain F52 to increase stand density and corn yield in fields, but wireworm control by *M.*

*anisopliae* was considered to be responsible factor (Kabaluk and Ericcson, 2007). Recently, *Metarhizium* spp. have also been reported to have antagonistic activity against some plant pathogenic micro-organisms, and the ability to endophytically colonize plant roots. We therefore could not rule out the possibility of these different mechanisms either alone, or in combination to be held responsible for plant growth promotion when *Metarhizium* is applied.

### **7.8. Root Rot of Beans**

Root rots are widely distributed and economically important diseases of the common bean, *Phaseolus vulgaris*. The disease incidence as well as severity of root rots and the damage induced, vary due to different micro-organisms involved in infection in addition to the environmental and soil conditions (Abawi and Corrales, 1990). If the environmental and soil conditions are favourable to the infection organism, the severity of damage increases. Damage is also high when crops experience stressful conditions such as drought, excess water, insect injury, or presence of other pathogenic organisms during the middle of the growing season. *Fusarium* species have been found to predominate on bean roots along with *Cylindrocarpon* spp. and members of the order *Mucorales* (Rovira, 1965). *Fusarium* species therefore hold an important place when bean root rot diseases of major importance are considered.

General symptoms and signs of damage by root-rot pathogens to beans include poor seedling establishment, stunting and uneven growth, leaf chlorosis (insufficient production of chlorophyll), premature defoliation, reduced yield, and premature death of severely infected plants, all of which reduce crop yield. However, root rot in beans very rarely shows uniform

infection throughout the field. Reduced plant vigour, uneven growth, and premature defoliation reduces crop yield.

*Fusarium* root rot is one of the most prevalent diseases of beans in the world. First recognised and reported in New York in 1919, *Fusarium* rot in beans is caused by the fungus *Fusarium solani* f. sp. *phaseolis*. This basidiomycetous, soil borne fungus survives in soil for a long time in the form of thick-walled spores called chlamydospores. It also produces thin-walled, single cell (microconidia) and multi-cell (macroconidia) spores. Such spores, if not converted to chlamydospores, are short-lived in soil. *F. solani* has been known to cause diseases in squash plants (Nawar, 2007), peanut (Widodo and Budiarti, 2009), soyabean, pea, and more. *F. solani* is capable of attacking plants singly and causes damage to bean plants exhibited as a distinct disease syndrome. However, synergistic interactions (where damage caused by two pathogens increases) do exist between *F. solani* f. sp. *phaseolis* and *Pythium ultimum*. The *Fusarium* root rot pathogen is also capable of surviving in soil by colonizing roots of non-host crops without causing disease.

Initial symptoms of *Fusarium* root rot appear about 1-2 weeks after planting, as longitudinal, narrow, bright-red streaks on hypocotyl and taproot surfaces, which increase with time. Infected areas become reddish brown and exhibit longitudinal fissures. In severe infection primary and lateral roots die and lower stem tissues become hollow and squishy. *F. solani* f. sp. *phaseolis* is capable of directly penetrating bean tissues and also invading the plant through wounds and natural openings.

*Trichoderma* species and particularly *Trichoderma harzianum* have been used as biocontrol agents against a variety of plant fungal pathogens (Lynch, 1987). *Trichoderma*

antagonistic activity has been observed against *Sclerotinia sclerotiorum*, *Fusarium culmorum*, and *Pythium ultimum* (Lynch, 1987).

## **8. THE Insect Pathogenic Fungus *Metarhizium robertsii* Is Also An Endophyte That Stimulates Plant Root Development**

### **8.1. Introduction**

The insect pathogenic fungus, *Metarhizium robertsii* J.F. Bisch., Rehner & Humber (Clavicipitaceae) is a common inhabitant of soils worldwide (Bidochka et al., 2001) and has been studied and used as an insect pathogen for biocontrol (Milner, 1997; Hunter et al., 2001; Lomer et al., 2001). However, this fungus appears to have, at least, a bifunctional lifestyle. Not only is it an insect pathogen but it also readily colonizes the plant rhizosphere (Hu and St. Leger, 2002). This bifunctional lifestyle is further exemplified in the differential expression of genes as an insect pathogen or in the presence of plant root exudate (Pava-Ripoll et al., 2011; Wang et al., 2005). For example, *M. robertsii* differentially expresses two adhesin genes, *MAD1* and *MAD2* (Wang and St. Leger, 2007). *MAD1* is used for adhesion to insect cuticles, and *MAD2* contributes to plant adhesion.

The utility of entomopathogenic fungi for pest control is a viable alternative to chemical insecticides. However, the ecological niche of *Metarhizium* in the soil is not well understood. It has been shown to be a rhizosphere competent fungus (Hu and St. Leger, 2002) but this association has yet to be fully elucidated. This association of *M. robertsii* with plant roots could prove to be an important aspect in the utilization of this fungus for insect control. Recently, several species of entomopathogenic fungi have been shown to play multiple roles in nature ranging from antagonists of plant pathogens to rhizosphere associates, endophytes, and possibly even plant-growth-promoting agents (Butt et al., 2001; Hu and St. Leger, 2002; Kabaluk and Ericsson, 2007; Ownley et al., 2008; Vega, 2008). Pyrenomycete fungi, to which *M. robertsii*

belongs, are a diverse class of filamentous ascomycetes that include insect pathogens (Moorhouse et al., 1993), plant pathogens (Freeman et al., 2000), decomposers (Samuels and Blackwell, 2001), fungal parasites (Spatafora and Blackwell, 1993), and human pathogens (Berbee and Taylor, 1992). Among them *Trichoderma*, *Beauveria* and *Lecanicillium* are insect biocontrol agents that are also endophytes (Ownley et al., 2010).

In this study we used light and confocal microscopy to elucidate root surface and endophytic colonization by *M. robertsii* of a grass species, switchgrass (*Panicum virgatum*), and a legume, haricot beans (*Phaseolus vulgaris*).

## **8.2. Materials and Methods**

### **8.2.1. Fungal cultures**

*M. robertsii* (strain Ma2575 = *Metarhizium anisopliae*; reclassified based on a multigene phylogenetic approach; Bischoff et al., 2009) obtained from the U. S. Department of Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, New York and a transformant of *M. robertsii* that expresses green fluorescent protein (*Metarhizium*-GFP) were used. The construction of the GFP-expressing plasmids, transformation and transgenic fungal lines has been previously described (Fang et al., 2006). The transformant did not show any differences in growth, insect virulence or colony morphology when compared to the wild-type. Another transformant of *M. robertsii*,  $\Delta$ *MAD2*, had an insertional disruption in the *MAD2* gene, which is required for the adhesion to plant cells, and was provided by Wang and St. Leger (2007). Stock cultures were grown at 27°C on potato dextrose agar (PDA; Difco Laboratories, BD, Mississauga, Ontario, Canada) adjusted to pH 6.9. Conidia were dislodged from the agar with 0.01% Triton X and the suspension passed through a funnel containing glass wool. The

conidial suspension was adjusted to  $1.5 \times 10^6$  conidia/mL using a haemocytometer and stored at 4°C for maximum two weeks.

### 8.2.2. Plant growth

We have previously shown that in Ontario, one genetic group of *M. anisopliae* (now *M. robertsii*) prefers agricultural habitats (Bidochka et al., 2001). Therefore for this study we investigated the association of the “*Metarhizium robertsii*” group of *Metarhizium* species with the roots of two agriculturally important plants. *Panicum virgatum* (switchgrass) and *Phaseolus vulgaris* (haricot beans) were obtained from OSC seeds (Waterloo, Ontario, Canada) and studied under axenic conditions. Seeds were surface-disinfected according to a modification of the method by Miche and Balandreau (2001). Seeds were soaked in sterile distilled water for 30 min in a 50 mL capped plastic tube. The seeds were then immersed in 4% sodium hypochlorite for 2.5 h. The fluid was decanted, and the seeds were rinsed with sterile distilled water. The seeds were then immersed in 15% hydrogen peroxide for 30 min and rinsed 3× with sterile distilled water. Axenically treated seeds were kept overnight at 4°C to allow for synchronization of growth. These were then used for respective experimentation. Axenically treated seeds were tested for fungal or bacterial contamination by plating onto PDA and nutrient agar.

### 8.2.3. Fungal inoculation of plants

We used two methods to observe the association of *M. robertsii* with plant roots. First, one method was based on the definition of “rhizosphere competence” of a biological control agent (Baker, 1991), where the biological control agent is capable of colonizing the rhizosphere of developing roots after seed treatment. Axenic seeds, whose germination was synchronized, were immersed into a fungal conidial suspension ( $10^6$  conidia/mL) for 1 h and placed onto

sterile, moist filter paper placed in Petri dishes and kept for up to 10 d with a photoperiod of 16 h (white fluorescent tubes, 8 h dark, 25°C). Sterile water was added as needed to keep the filter paper moistened. A control group was not inoculated with fungal conidia but was immersed in sterile deionised water containing 0.01% Triton-X collected from a PDA plate in the same manner as conidia were dislodged, to mimic the conditions of the treated seeds. Ten treated seeds of switchgrass per dish and one treated seed of bean per dish were used. For experimental manipulations and controls, two experimental replicates were used with five Petri dishes in each replicate. Root lengths of switch grass seedlings were measured for 5 d after seed germination. This method was used to observe whether *Metarhizium* colonized the plant root after seed inoculation. Root hair density was measured using a dissecting microscope (Leica ES2) for randomly selected switchgrass roots and measured as the number of root hairs per millimeter of root section at several positions along the root length.

In the second method, soil instead of filter paper was used to investigate fungal associations with seedlings of haricot beans. This method was used to analyse whether the rhizosphere-competent entomopathogenic fungi present in soil are able to colonize the rhizosphere of developing roots under sterile and non-sterile soil conditions. Soil (obtained from Schultz Potting Mixture, Brantford, ON, Canada) was sterilized by autoclaving at 121°C for 1 h followed by a 30-min liquid cycle. Petri dishes were filled with the soil (sterile or non-sterile) and moistened with sterile distilled water. The soil was then inoculated with three square agar plugs (1 cm<sup>2</sup> each) from an actively growing colony of the respective fungal cultures. Plugs were placed with the growing side of the agar plug towards the soil surface, and the plates were then incubated at 27°C for 10 d. Controls contained agar plugs without fungal conidia. Sterile soil samples were plated onto PDA to monitor any microbial contamination before and after



experimentation. After 10 d, a fungal mass was visible over the soil surface and within soil cavities. Next, the Petri dish was opened, and the side wall of the Petri dish was punctured with a hot scalpel at one point. The root of a single seedling (previously germinated for 10 d and axenic) was then inserted through this hole such that the leaves remained outside of the Petri dish. The Petri dish was closed and covered with aluminum foil to keep the roots in darkness to mimic hypogeous conditions, and 25 replicates were plated for each treatment with one seedling per Petri dish. These Petri dishes were then incubated vertically in a chamber with photoperiod of 16 h (white fluorescent tubes, 8 h dark, 25°C) for up to 60 days (Kottke et al., 1987). Plants were monitored regularly and moistened with sterile distilled water as required. Lateral root emergence was assessed for randomly selected bean seedlings as the number of new lateral roots that emerged after axenically grown seedlings were transplanted into growth chambers. Plant conditions were monitored every day for up to 60 d. Statistical *t* tests were used to compare mean values of root lengths, root hair density and lateral root emergence.

A confirmatory test was conducted to recover *Metarhizium* from bean roots. *Metarhizium* was isolated from the roots by a modified method of Wyrebek et al. (2011) where roots washed with sterile water were cut into 0.5 cm pieces. These were then surface sterilized by immersion in 4% sodium hypochlorite solution for 5 min and homogenized using a rotary homogenizer (Greiner Scientific, Frickenhausen Germany). One hundred microliters of this homogenate was then plated onto selective PDA media (containing 0.5 g cyclohexamide/L, 0.2 g chloramphenicol/L, 0.5 g 65% dodine/L, and 0.01 g crystal violet/L) and incubated at 27°C for 20 d. *Metarhizium* isolates were identified based on growth on selective media, colony morphology and microscopic identification of conidia.

#### 8.2.4. Microscopy: Light, Fluorescence and Confocal

The associations between *Metarhizium* and plant roots were observed microscopically. Initially, roots were examined with a scanning electron microscope (SEM). Roots were dehydrated through a graded ethanol series and mounted on aluminum mounts, using double-sided carbon tape, and coated with gold/palladium (Higashi and Abe, 1980). The sections were examined with an SEM (AMRAY 1600T) (Bedford, Massachusetts, USA) at 10-15 kV.

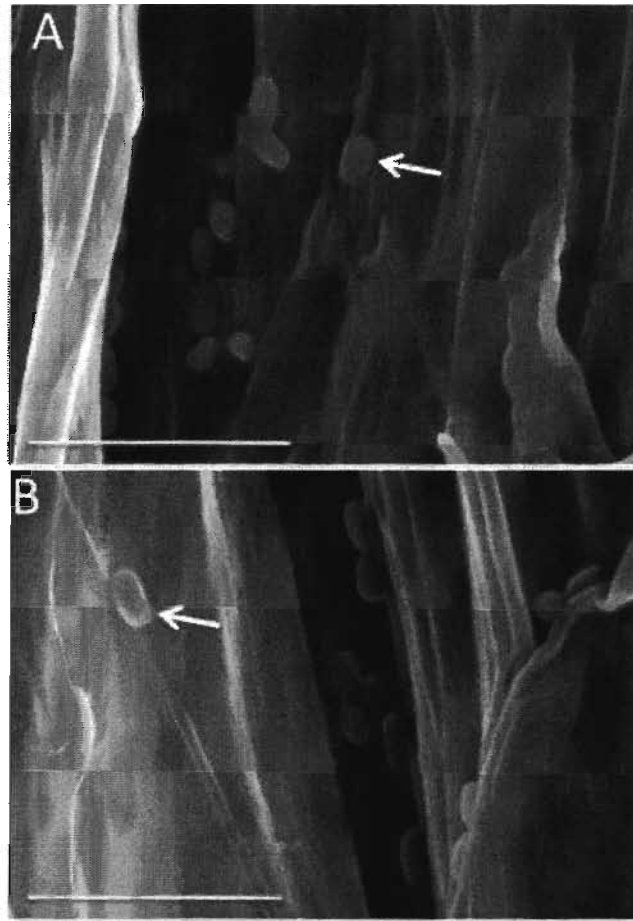
Whole roots of switchgrass as well as of haricot bean were mounted on glass slides for light and confocal microscopic examination. Light microscopy was also used to observe and quantify differences in root hair densities in the control and treated plants. Also after 60 d, whole root sections as well as longitudinal sections of haricot bean were examined. Five root sections per replicate were randomly selected and examined. Roots obtained after 60 d of associations were washed thoroughly with sterile distilled water to remove any adhered soil. These were then cut to a length of 1-2 cm and cleared by either boiling in 10% KOH for 60 minutes at 90°C or keeping the roots in 10% KOH for 10 d at room temperature followed by a wash with sterile distilled water. Roots were then immersed in 70% ethanol for 5 min and stained with lactophenol cotton blue for 5 min followed by a destain in 50% glycerol (Chilvers et al., 1987). Longitudinal sections were examined using a light microscope. For confocal microscopy whole root sections without any clearing and staining were examined.

Tissues were examined with a Leitz Diaplan light microscope and photographed using Leica DFC-400, Microsystems and/or Leica DM RBE laser scanning confocal microscope (LSCM) equipped with an argon-krypton laser operated at excitation wavelengths of 480±10 nm and 518nm.

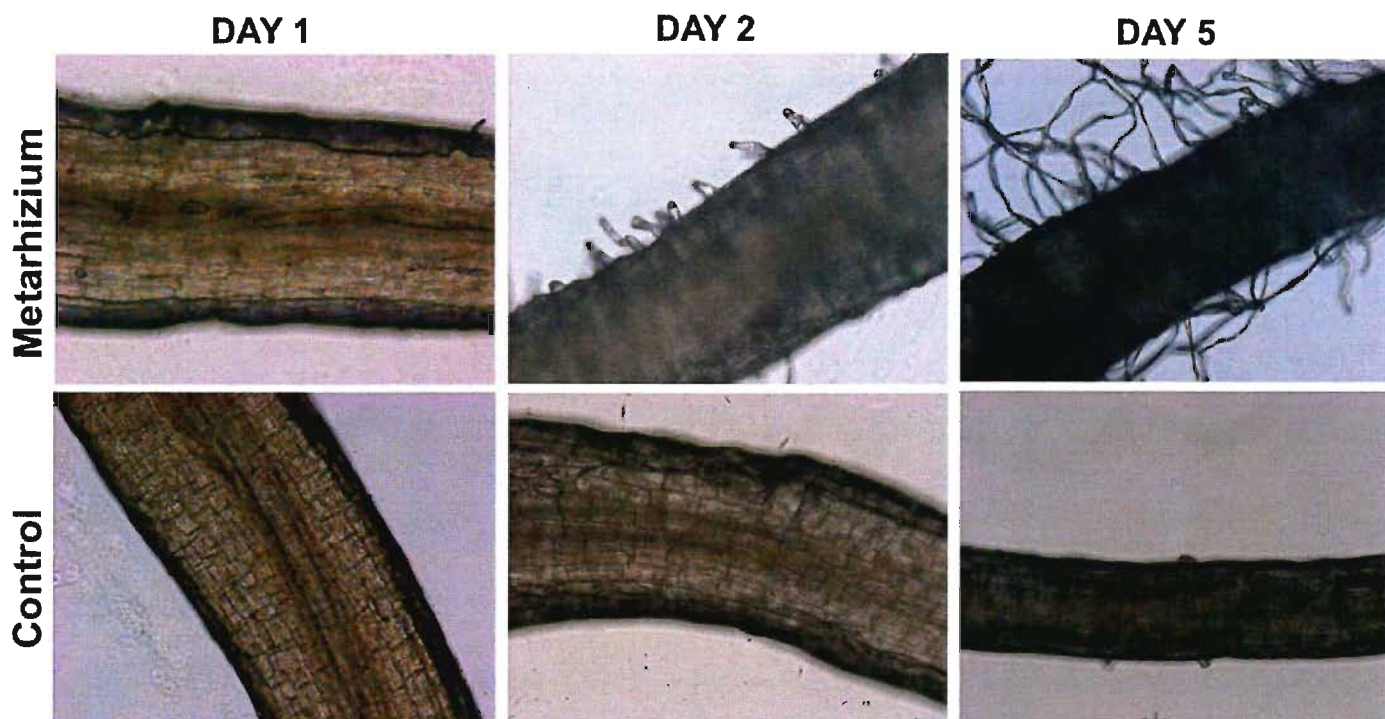
## 8.3. RESULTS

### 8.3.1. Root colonization by *Metarhizium* and stimulation of root and root hair development

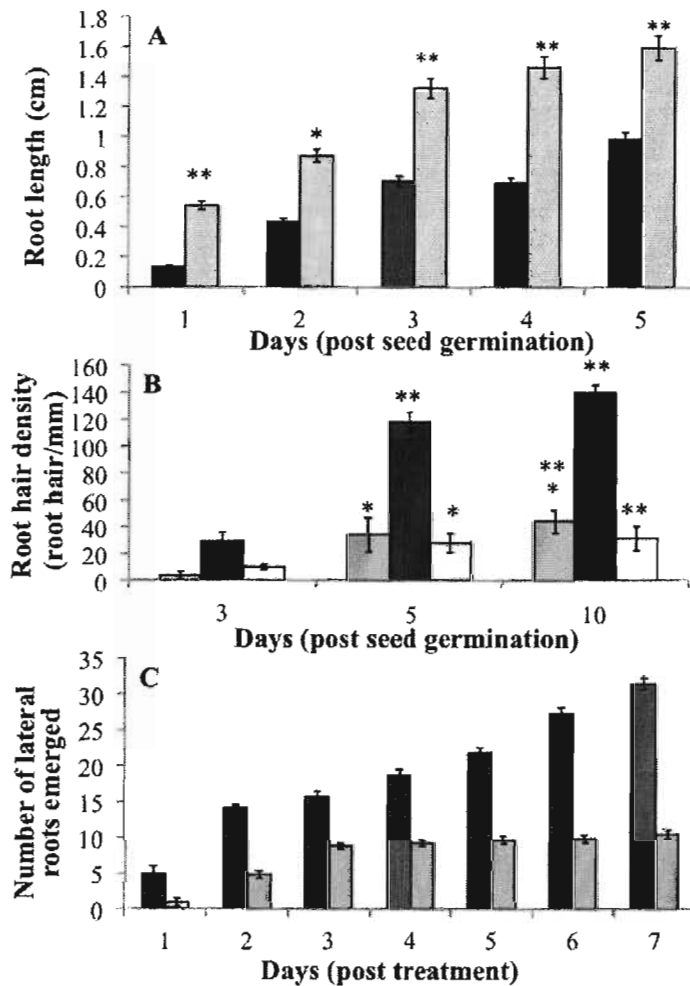
Initial experiments showed that *M. robertsii* conidia adhered to switchgrass roots (Fig. 3). Compared to control roots, switchgrass treated with *M. robertsii* showed a proliferation of root hairs. Differences in the appearance of root hairs could be seen between the control and the *M. robertsii*-treated roots even after 2 d after seed germination (Fig. 4). Not only do plants treated with *M. robertsii* initiate root hair development earlier than control plants, but the root lengths of plants treated with *M. robertsii* were significantly longer (*t*-test:  $t = 10.7504$ ,  $df = 48$ ,  $P < 0.0001$  on day 5) than control roots (Fig. 5A). Differences in root hair density were also observed when switchgrass was treated with *M. robertsii* (Fig. 5B). Root hair density for control and  $\Delta MAD2$ , respectively, were significantly lower than *M. robertsii*-treated seedlings on day 3 after seed germination (*t*-test:  $t = 13.43$  and  $8.62$ ,  $df = 10$ ,  $P < 0.0001$ ). This trend continued 10 d after germination as well, with lower root hair density for control and  $\Delta MAD2$ -treated plants, respectively, as compared to *M. robertsii*-treated seedlings (*t*-test:  $t = 17.88$  and  $26.31$ ,  $df = 10$ ,  $P < 0.0001$ ). Also, treatment of bean roots with *M. robertsii*, showed greater emergence of lateral root hairs as compared to control (Fig. 5C). Treatment with *M. robertsii* significantly increased lateral root emergence in bean seedlings as compared with control (*t*-test:  $t = 20.03$ ,  $df = 12$ ,  $P < 0.0001$ ). Plants treated with *M. robertsii*  $\Delta MAD2$  showed very little root hair proliferation after 10 d. Furthermore, fewer  $\Delta MAD2$  conidia adhered to plant roots. Plant roots treated with cell free extracts of *M. robertsii* grown in liquid broth (YPD) for 3 d did not show root hair proliferation (data not shown). We found no differences in root hair density or lateral root emergence with plants treated with *Metarhizium* under sterile or non-sterile conditions.



**Figure 3.** Adhesion of conidia of *Metarhizium robertsii* to plant root wall (arrows). (A, B) Scanning electron micrographs of switchgrass roots 1 h. after inoculation with conidia. Bar = 50 $\mu$ m.



**Figure 4.** Light micrographs showing the time course (in days) of root hair development in switch grass seedlings treated with *M. robertsii* and control. Superficially disinfected and synchronised seeds were treated with either no conidia for control or a conidial suspension ( $10^6$  conidia/mL 0.01% Triton X) of *M. robertsii*. Day 1 = first day after conidial treatment.

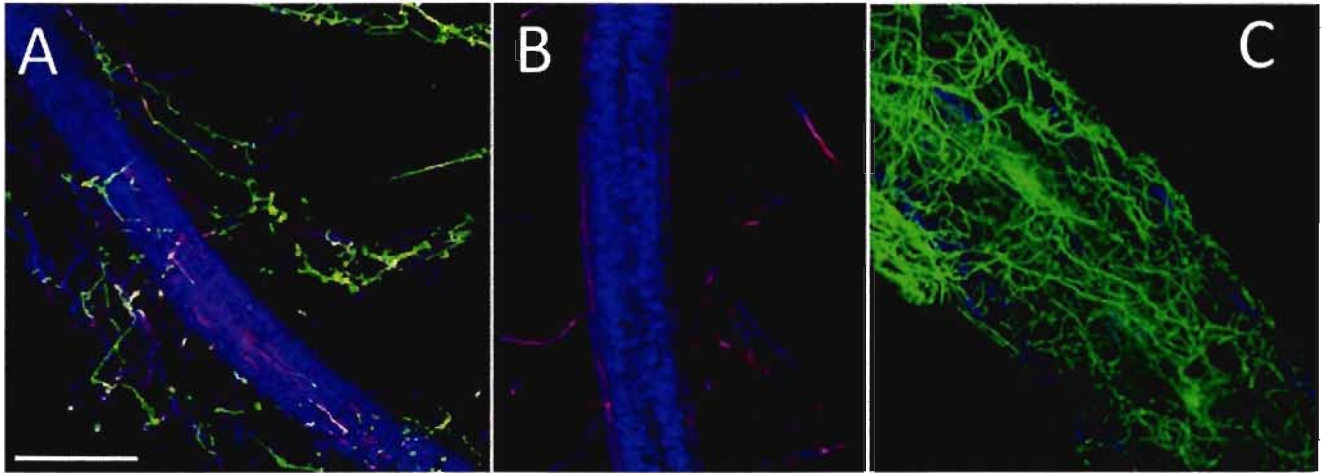


**Figure 5.** (A) Time course of root length growth for control (dark bars) and *M. robertsii*-treated (light bars) switchgrass up to 5 d post germination. Each column represents the mean  $\pm$  SE of root lengths for control and treated plants which differed significantly at each day (Student's *t*-test:  $N=50$ , \*\*  $P<0.0001$ , \*  $P<0.01$ ). (B) Time course of root hair density for control (grey bars), *M. robertsii*-treated (black bars) and  $\Delta$ MAD2-treated (white bars) switchgrass up to 10 d post germination. Each column represents the mean  $\pm$  SE of root hair density measured for control and treated plants where the *M. robertsii* treated seeds showed significantly greater root hair density at days 5 and 10 than control or  $\Delta$ MAD2-treated seeds (*t*-test:  $N=11$ ,  $P<0.01$ ), while no differences were observed between control or  $\Delta$ MAD2-treated seeds (*t*-test:  $P>0.05$ ). (C) Time course of lateral root emergence for *M. robertsii*-treated (dark bars) and control (light bars) bean roots up to 8 d post treatment. Each column represents the mean  $\pm$  SE of the number of lateral roots counted, which differed significantly at each day (*t*-test:  $N=7$ ,  $P<0.0001$ ).

Confocal micrographs supported the association of *Metarhizium*-GFP with plant roots of switchgrass, which could be seen as green-fluorescing mycelia (Fig. 6A). However, the same was absent in control sections (Fig 6B). Also, note that in Figure 4A the proliferation of roots hairs in the *M. robertsii*-treated plants when compared to the control plants. The hyphae of *Metarhizium*-GFP could also be seen surrounding the bean root surfaces 10 d post germination in soil as well as on filter papers (Fig. 6C). The root hair density and lateral root emergence were observed for the initial period of association between *M. robertsii* and plant roots.

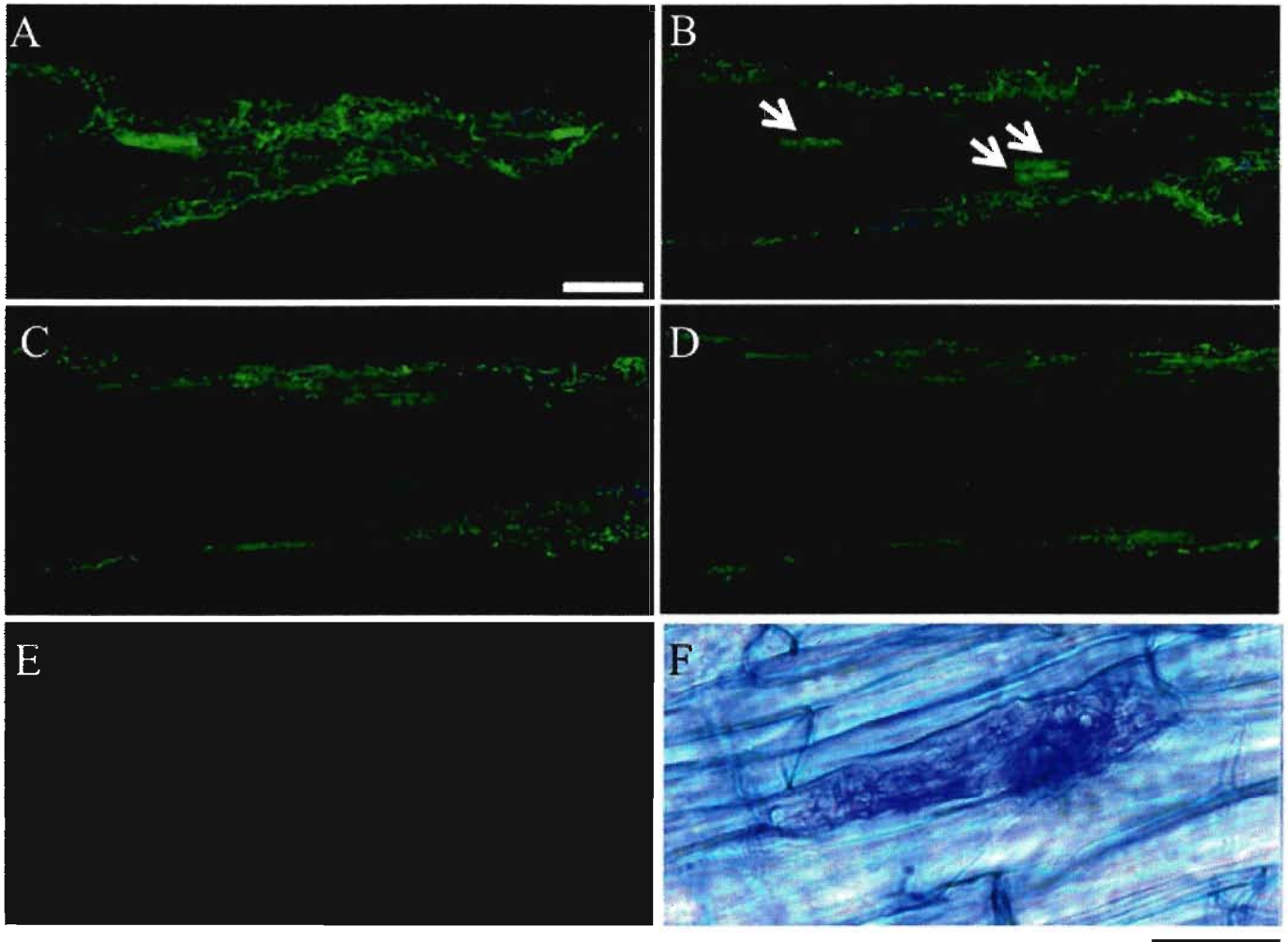
### 8.3.2. Endophytic association of *Metarhizium* in soil

Bean seedlings were grown in soil, with or without *M. robertsii*-GFP for up to 60 d. To confirm an endophytic association, we observed GFP fluorescence in z-stacks of the whole root using confocal microscopy. GFP-expressing *Metarhizium* could be observed on the surface of bean roots (Fig. 7A). As the root was sequentially microphotographed (sequential 18.4  $\mu\text{m}$  depths), several cortical cells were observed to be colonized by *Metarhizium*-GFP (Fig. 7 B-D) suggesting endophytic colonization by *M. robertsii*. A negative control, with non GFP *Mr*-2575 was also visualised using z-stacks, and by the absence of the green signal (Fig. 7E), the GFP-signal was discerned from laser noise. Nor was the GFP-signal observed in the control plants. GFP-expressing *Metarhizium* was also observed to colonize plants in non-sterile soil. To further investigate the colonization of *M. robertsii* in the cortical cells, we examined tissue sections of plant roots cleared with KOH and stained with lactophenol cotton blue. *M. robertsii* penetrated the bean root cell wall within 60 d, invaded the root cells, and grew within intracellular spaces



**Figure 6.** Confocal scanning electron micrographs of whole roots treated with *Metarhizium*-GFP. (A) A section of switchgrass whole root treated with *M. robertsii* showing associated GFP-expressing hyphae. Also, note the proliferation of root hairs. (B) Section of control switchgrass whole root. Blue is plant autofluorescence. Root sections were obtained 10 d post germination. (C) Whole root section showing *M. robertsii* growing on the surface of bean root 10 d post germination. Bars=200 $\mu$ m.





**Figure 7.** Confocal scanning electron micrographs obtained after 60 d of interaction between bean roots and *M. robertsii*. (A) surface of bean roots showing *M. robertsii* hyphae on the surface of bean root. (B), (C) and (D) are z-stacks obtained from the same root with step size=18.4 $\mu$ m and show endophytic colonization of bean root cortical cells by green-fluorescing *M. robertsii* (see arrows in panel B) (E) No GFP signal was seen in the cortical cells of bean root treated with non-GFP *M. robertsii*. (F) Light micrograph of cleared bean root, longitudinal section, stained with lactophenol cotton blue, showing mycelial mass within root cortical cell. Bars=200 $\mu$ m.

and within the root cells. The fungus could be observed as a mycelial mass within individual cortical cells (Fig. 7F). *Metarhizium robertsii*  $\Delta$ MAD2 was also found to associate with plants after 60 d (data not shown). Sections containing *M. robertsii*-GFP cleared with KOH did not show a GFP signal. Also, *Metarhizium* was recovered from root homogenate plated on selective PDA.

#### 8.4. Discussion

Here we investigated the colonization (for up to 60 d) by *M. robertsii* of roots of two plants of agricultural importance: switchgrass and haricot bean. Switchgrass is being developed as a renewable biofuel (Sanderson et al., 1996, 2006). We demonstrated that *M. robertsii* (1) was intimately associated with plant roots, (2) induced root hair proliferation and plant root growth when compared to control plants, and (3) resided as an endophytic colonizer in beans.

*Metarhizium robertsii*  $\Delta$ MAD2 strain had reduced adhesion to plant roots, delayed colonization, and endophytic growth, suggesting that MAD2 is not solely responsible for root colonization and endophytic potential.

The stimulation of root hair development and lateral root formation by *M. robertsii* suggested that *M. robertsii* is a plant-growth-promoting fungus. Stimulation of lateral root development is considered an early phase of interaction in non-phytopathogenic, root-colonizing fungi (Felten et al., 2009). Increased root branching has been observed as a response to colonization by some species of rhizosphere-inhabiting fungi (Harrison, 2005). The endophytic fungus EF-37 was associated with the roots of *Saussurea involucrata* (snow lotus), promoted the growth of plant roots, and increased the number of root hairs (Wu et al., 2010). *Trichoderma*-inoculated maize roots were deeper, more robust and had greater surface area (Harman et al.,

2004). The basidiomycete *Piriformospora indica*, endophytically colonized roots and promoted root growth in a number of plant species (Varma et al., 1999). We observed increased root hair development in switchgrass before notable colonization by *Metarhizium*, a phenomenon also observed in *Arabidopsis* roots inoculated with *P. indica* (Peskan-Berghofer et al., 2004).

*Metarhizium* is added to a list of fungi that have bifunctional lifestyles as an insect pathogen as well as a plant endophyte. Many fungi traditionally known as insect pathogens have also been shown to be endophytes, including species of *Acremonium*, *Beauveria*, *Cladosporium*, *Clonostachys* and *Isaria* (Vega, 2008). Other insect pathogenic fungal species documented to have endophytic colonization potential include *Lecanicillium* (Ascomycota, Hypocreales) and *Trichoderma* (Ascomycota, Hypocreales) (Ownley et al., 2010). In a recent comparative transcriptomics study, *Metarhizium* shared 16% identity with plant endophytes and plant pathogens (Gao et al., 2011). A Finnish isolate (V245) of *M. anisopliae* has been reported to penetrate some leaf surfaces after conidia germination (Inyang et al., 1996). Moreover, *Metarhizium* may have evolved subtle ecological adaptations to insect parasitism in the soil (Vega et al., 2009). The jump to new hosts sharing a habitat (i.e., plants roots and soil-inhabiting insects) has been speculated among other members of the Hypocreales (Spatafora et al., 2007; Vega et al., 2009). *Metarhizium* (also a Hypocreales) could have made the shift from a plant-root associate to an insect pathogen. The reverse may also be true, that the ancestral lifestyle of *Metarhizium* was as an insect pathogen followed by adaptations to the rhizosphere. However, *Metarhizium* shares a phylogenetic basal root with the fungal grass-endosymbionts *Claviceps* and *Epichloë* (Spatafora et al., 2007) and suggests that the ancestral lifestyle was as a plant associate.

This study showed that *M. robertsii* is a plant endophyte with plant-root-promoting properties. Further research is needed to confirm the role of *M. robertsii* and the coordinated communication between the fungus and plant. What we do know is that *M. robertsii* cannot be defined simply as an insect-pathogen. We have been investigating the utility of these fungi as insect pathogens, and perhaps we can say with some assurance that there is more going on underground with *M. robertsii* and plants and the promotion of plant growth and potentially, plant protection, than we may have previously suspected. *Metarhizium robertsii* is a fungus that could be used in multiple roles, from protecting plants from pests to promoting plant growth.

## 9. Antagonism of *Metarhizium robertsii* against the bean plant pathogen *Fusarium solani* f. sp. *phaseolis*.

### 9.1. Introduction

*Phaseolus vulgaris* (haricot beans) are highly susceptible to root rot disease caused by the soil borne fungal pathogen *Fusarium solani* f. sp. *phaseolis*. (Steadman et al., 1975; Graham, 1978; Tan and Tu, 1995; Cichy et al., 2007). Disease symptoms are initially characterised by narrow, long, red to brown lesions on stems extending down to the main taproot which may decay and die. In some cases, the plant hypocotyl is also affected along with the development of lateral or adventitious roots above the damaged taproot. Infected plants also show stunted growth when compared with healthy plants (Burke and Barker, 1966; Campbell, 1989; Schneider and Kelly, 2000; Cichy et al., 2007).

Fungicides are used to control *F.solani* root rot (Campbell, 1989). Spraying crops with fungicides is not always successful, and may lead to fungicide resistance of pathogen populations, environmental contamination, harm to human health, and high costs. In this context, biological control appears to be an effective alternative and a number of pathosystems are currently available. *Trichoderma* (El- Kassa and Khairy, 2009; Rojo et al., 2006; Grosch, 2006;), *Pythium oligandrum* (Floch et al., 2005), and other fungal species are reportedly used as biocontrol agents of plant root diseases.

*Metarhizium robertsii*, a well-known insect pathogenic fungus, has recently been shown to be a plant endophyte and plant root growth promoter (see chapter 8). *Metarhizium spp.* have also previously been shown to possess antifungal properties against *Fusarium oxysporum*, *Botrytis cinerea*, and *Alternaria solani* (Kang et al., 1996) and *Ceratocystis ulmi* (Gemma et al.,

1984). Here the possibility that *M. robertsii* could be antagonistic against the bean root rot pathogen *F. solani* f. sp. *phaseolis* under in vitro conditions and in vivo conditions have been explored.

## **9.2. Materials and Methods**

### **9.2.1. Fungal isolates**

*M. robertsii* (ARSEF 2575) was obtained from the United States Department of Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, New York. Cultures were grown on potato dextrose agar (PDA; Difco Laboratories, BD, Sparks, MD, USA) at 27°C for 10 d to obtain conidia. *F. solani* f. sp. *phaseolis* (DAOM 170970) was obtained from the Canadian Culture Collections, Agriculture Canada, Ottawa. This strain was isolated from infected bean roots. *Fusarium oxysporum* was obtained from Wards (St. Catharines, ON), and originally isolated from infected wheat roots. *F. solani* and *F. oxysporum* were grown on PDA for 14 d at 25° C to obtain macroconidia. Conidia for all cultures were harvested into 0.01% (v/v) Triton X-100. The conidial suspensions were filtered through glass wool to remove mycelia, and adjusted to 10<sup>6</sup> conidia/mL using a haemocytometer (Brightfield). All the working conidial concentrations were adjusted using sterile distilled water.

### **9.2.2. Antagonism in plate assays**

The antagonistic activity of *M. robertsii* against *F. solani* was screened using dual culture plate assays (Sobowale et al., 2010). *M. robertsii* and *F. solani* were inoculated 3 cm apart from each other and from the edge of the Petri dish (9 cm diameter) containing PDA. *M. robertsii* was inoculated first, and allowed to grow for two days before the introduction of *F. solani*. Controls contained *M. robertsii* or *F. solani* alone, in order to observe the growth in the

absence of interactions. Additionally, controls with *F. solani* and *F. oxysporum* inoculated 3 cm apart from the edge of the plate were utilized. Fungal growth was observed for 14 d at 25°C. Growth of *F. solani* was measured as the average diameter of the colony size in four different directions. *F. solani* growth on day 8 was used for data analysis. The percent inhibition in the radial colony growth was calculated as per Mishra (2010); where percent inhibition was calculated as  $[(C-T)/C] \times 100$ , where, C is the radial growth in control set and T is the radial growth in treated set.

The formation of clearing zones by *M. robertsii* in the presence of *F. solani* was investigated by introducing *M. robertsii* on a *F. solani* lawn culture that was grown for 5 d. *M. robertsii* was inoculated (10 µL of  $10^8$  conidia/mL) at four different points on the *F. solani* lawn. Plates were incubated at 25°C for 14 d and clearing zones obtained by *M. robertsii* were measured on day 7 and 14. Controls contained a *F. solani* lawn inoculated with *M. robertsii* cell-free culture filtrates.

### **9.2.3. *Metarhizium robertsii* cell free culture filtrate**

The cell-free culture filtrate of *M. robertsii* was tested to (i) show the effect on germination of *F. solani* and (ii) to show the effect on growth of liquid culture of *F. solani*. To obtain the culture filtrates *M. robertsii* was inoculated into 100 mL sterilised water yeast extract broth (0.1% yeast extract in distilled water; WYEB), and incubated at 25°C. Culture filtrates were obtained on day 4 and 10 of fungal growth by vacuum filtration through Whatman filter paper no. 44 followed by filtration through 0.22 µm Millipore membrane. The cell free culture filtrates were diluted with WYEB, and 3 mL of 100%, 90%, 50% and 10% (v/v) cell-free culture filtrate were inoculated with 300 µL of a conidial suspension ( $10^8$  *F. solani* conidia/mL) in glass

tubes. WYEB was used as the control. The conidial suspensions were incubated at 25°C. The number of *F. solani* conidia germinated per hundred cells was counted at 24 and 48 h. Four readings were taken from each treatment and the experiment was repeated in triplicate.

The effect of *M. robertsii* cell free culture filtrates on *F. solani* growth was also observed for a 4 day period. Autoclaved, as well as non-autoclaved, *M. robertsii* cell free culture filtrate was obtained after 4 d and 10 d of *M. robertsii* growth as described above. *F. solani* conidial suspension (1.25 mL of  $10^8$  cells/mL) was added to 125 mL of *M. robertsii* 100% cell-free culture filtrate in glass flasks. For controls, WYEB was used. Flasks were placed on a rotary shaker at 150 rpm at 25°C for 4 d. The cultures were harvested by vacuum filtration through Whatman No. 44 filter paper, dried at 40°C for 48 h, and weighed.

#### **9.2.4. In vivo assays**

Haricot bean (*Phaseolus vulgaris*) was used to study the antagonistic effect of *M. robertsii* on bean root rot pathogen *F. solani*. Bean seeds; obtained from OSC seeds (Waterloo, Ontario, Canada) were surface disinfected prior to using a modification of the method of Miche and Balandreau (2001). Seeds soaked in sterile distilled water for 30 min were immersed in 4% sodium hypochlorite for three 10 min washes followed by a single 30 min wash with 30% hydrogen peroxide. Seeds were then washed with sterile distilled water and kept overnight at 4°C to allow for synchronization of growth. These axenically treated seeds were tested for fungal and bacterial contamination by plating on PDA and nutrient agar.

The effect of pretreating soil (obtained from Schultz Potting Mixture, Brantford, ON, Canada) with *M. robertsii* as an antagonist against *F. solani* disease in bean plants was investigated in growth chamber experiments. Soil was moistened with sterile distilled water and



autoclaved in trays (dimensions: 3 cm X 19 cm X 28 cm) twice before use. Eight plugs (1 cm<sup>2</sup> each) from an actively growing fungal colony were mixed in soil and incubated for 10 d at 25°C. Soil was mixed every alternate day to ensure uniform inoculation. Different treatments of soil were used for biocontrol assays: (1) without any treatment, (2) with *M. robertsii*, (3) with *F. solani*, and (4) with *M. robertsii* and *F. solani* (1:1 ratio). Bean seeds were placed in the upper 0.5 cm of soil for each treatment. Soils were watered with half strength MMN medium [CaCl<sub>2</sub> 0.05g, NaCl 0.025g, KH<sub>2</sub>PO<sub>4</sub> 0.5g, (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> 0.5g, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.15g, FeCl<sub>3</sub>.6H<sub>2</sub>O 1 mg, thiamine hydrochloride 100 µg, glucose monohydrate 5g, stock solution of trace elements (stock solution of trace elements: KCL 3.728g, H<sub>3</sub>BO<sub>3</sub> 1.546g, MnSO<sub>4</sub>.H<sub>2</sub>O 0.845g, ZnSO<sub>4</sub>.7H<sub>2</sub>O 0.575g, CuSO<sub>4</sub>.5H<sub>2</sub>O 0.125g, (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.4H<sub>2</sub>O 0.018g, per litre) 10 mL/L., Kottke et al., 1987] once a week and plants were watered daily (with sterile distilled water) to maintain soil moisture. Plants were maintained in a growth chamber (25/20°C, 60/80% relative humidity and 16/8 h day/night cycle; Grosch *et al.*, 2006) for 4 weeks. Each treatment included four replicates with five plants each. Different plant growth parameters such as number of seedlings emerged, shoot height, number of leaves/ plant, plant health, and any visible disease symptoms were assessed every day. After 4 weeks, whole plants were removed from the soil and roots were rinsed of soil with sterile water. Root and shoot fresh weight, height, dry weight and number of leaves were measured for individual plants in all treatments. Plants were individually rated for disease severity based on root rot index and rot color intensity according to Dar et al. (1997). Individual plants were rated on a scale of 0-1, where 0 = no root rot symptoms; 0.10 = less than 10% root area rotted; 0.25 = 11-25% root area rotted; 0.50 = 26-50% root area rotted; 0.75 = 51-75% root area rotted; and 1.00 = more than 75% root area rotted. The individual ratings were converted to a mean rot index by taking the quotient of the sum of the individual plant rating

values and the number of plants assessed. Plants were also rated for disease severity based on necrotic lesions on the roots and hypocotyl as described by Filion et al. (2003). The ratings were made on a scale of 0-5, where 0 = no disease symptoms, 1 = slightly brown or <50% surface discoloration of the hypocotyl, firm upon pressure from thumb and forefinger, and slight root pruning; 2 = as 1 but >50% surface discoloration; 3 = discoloured hypocotyl and roots collapsing under considerable pressure and extensive root pruning, 4 = darkly discolored hypocotyl and roots completely collapsed or collapsing easily under pressure and severe root pruning; and 5 = dead or dying plant.

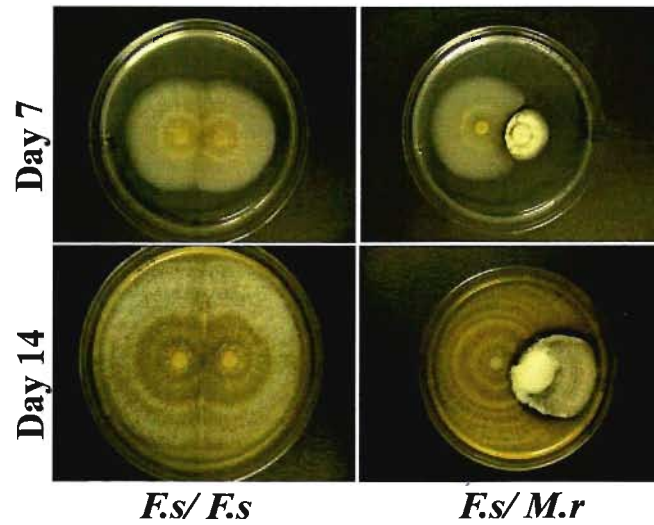
### **9.3. Results and Discussion**

#### **9.3.1. Antagonism of *M. robertsii* to *F. solani***

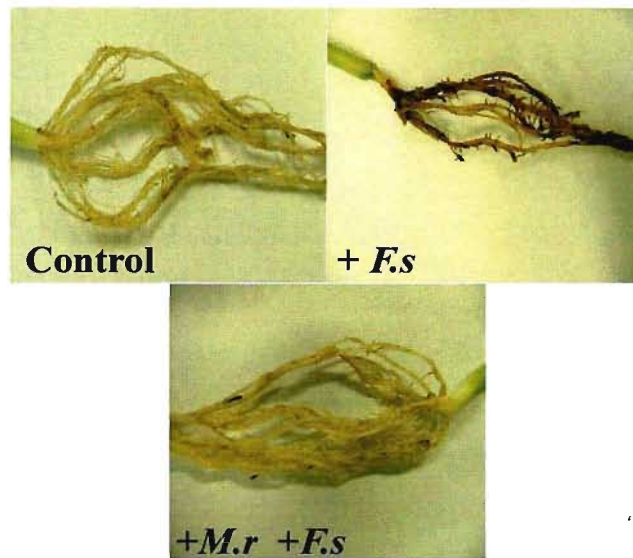
*M. robertsii* was initially screened using plate assays to analyse for any potential antagonistic activity against *F. solani*. When *F. solani* and *M. robertsii* were co-cultured on Petri dishes, an inhibition clearing zone could be seen on day 4 and this effect was observed up to day 14 (Fig 8A). Clearing zones were also absent when *F. oxysporum* and *F. solani* were co-cultured (data not shown).

Inhibition of *F. solani* growth by *M. robertsii* was also measured as a decrease in radial growth. A relative inhibition of 59.4 % of *F. solani* growth was observed when co-cultured with *M. robertsii*. The zone of *F. solani* inhibition of  $3.7 \pm 0.5$ mm and  $5.9 \pm 1.1$  mm on a lawn culture was observed at days 7 and 14, respectively, by *M. robertsii*. Furthermore, *M. robertsii* could be observed overgrowing *F. solani* in dual culture plate assays and white mycelial growth of *M. robertsii* could be seen at the interface with *F. solani*. The darker zone of confluent growth could be indicative of melanization or necrosis of *F. solani* hyphae.

A.



B.



**Figure 8.** Images showing antagonistic activity of *Metarhizium robertsii* on *Fusarium solani*. (A) Dual plate assays showing antagonistic activity of *M. robertsii* on *F. solani* on day 7 and 14. Zones of *F. solani* clearing is observed at the colony interface. (B) Images of bean root rot conditions caused by *F. solani* after 4 weeks. Roots obtained from control plants, *F. solani* treatment and *M. robertsii* + *F. solani* treatments.

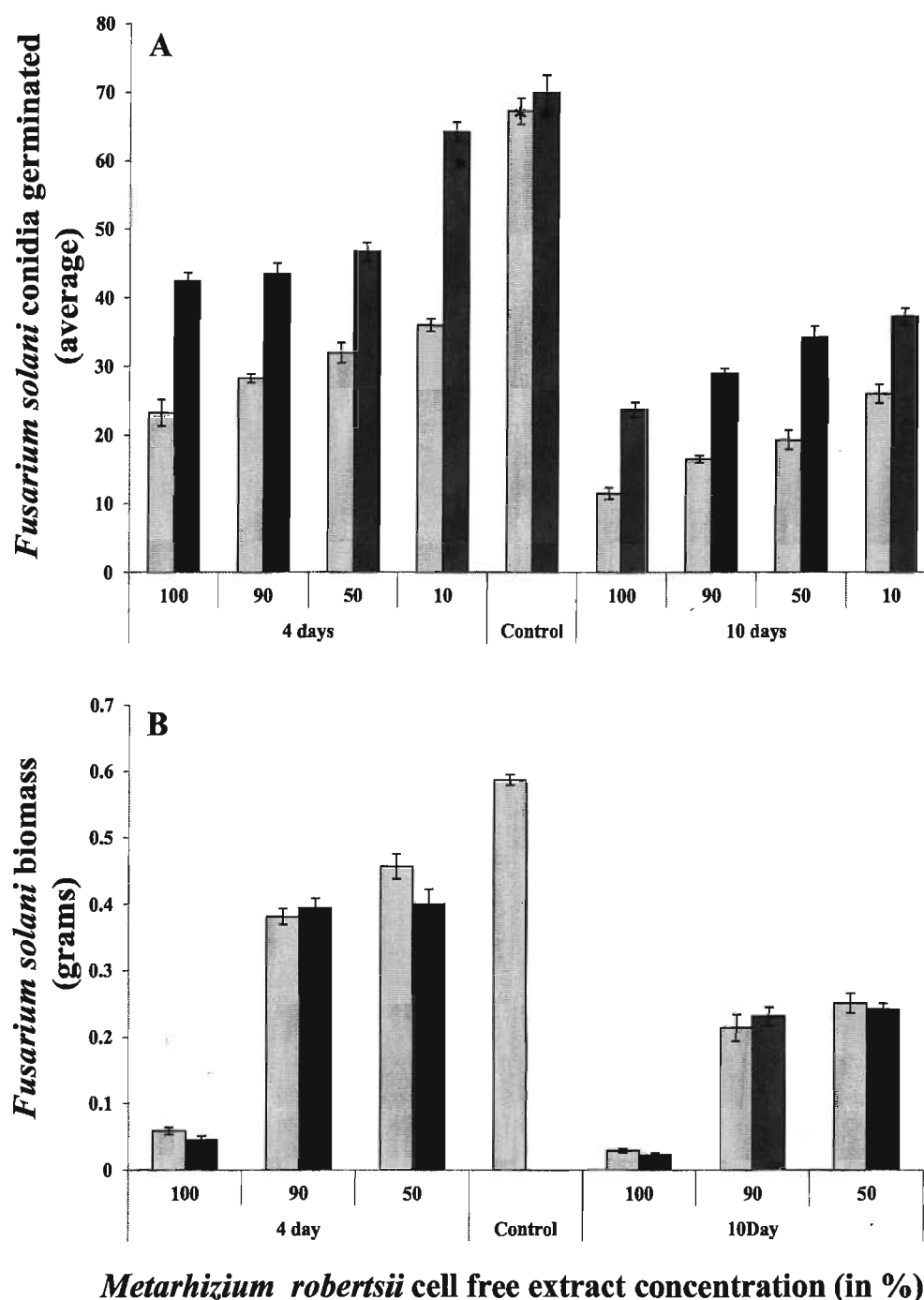
### 9.3.2. Inhibition of *F. solani* conidial germination by *M. robertsii* cell free culture extracts

*F. solani* conidia incubated in cell free culture filtrates of *M. robertsii* showed delayed germination (Fig. 9A). *F. solani* conidia incubated in 100% *M. robertsii* cell free culture extract collected on day 10, resulted in 83% and 66% germination inhibition at 24 and 48 h, respectively ( $t$  test:  $P < 0.0001$ ,  $t = 26.85$ ). In 4 d *M. robertsii* cell free extract germination inhibition was 65% and 39% at 24 and 48 h, respectively ( $t$  test:  $P < 0.0001$ ,  $t = 16.29$ ). These results support that *M. robertsii* secrete compounds that inhibits *F. solani* germination. Additionally, the amount of the compound secreted by *M. robertsii* increased in older cultures.

*M. robertsii* cell free culture filtrates also decreased *F. solani* vegetative growth (Fig. 9B). Autoclaving the cell free culture filtrate did not reduce the inhibitory effect on *F. solani*. No significant difference was observed in *F. solani* biomass obtained in autoclaved compared to not-autoclaved *M. robertsii* cell free culture filtrates ( $t$  test:  $P > 0.05$ ,  $n = 10$ ). This suggests that the inhibitory compound(s) are heat stable. *F. solani* biomass obtained in control cultures was significantly higher ( $t$  test:  $P < 0.0001$ ,  $n = 10$ ) than without *M. robertsii* cell free culture filtrate.

### 9.3.3. In vivo antagonism of *M. robertsii* against *F. solani*

Bean plant bioassays were performed in presence of *F. solani* and *M. robertsii*. While all seeds emerged into healthy seedlings by day 11 in the control and *F. solani* + *M. robertsii* treatment, only 60% seeds emerged in the presence of pathogen alone. The remaining 40% seeds were infected by *F. solani* even before they could germinate.



**Figure 9.** Inhibition of *Fusarium solani* in *Metarhizium robertsii* cell free culture extracts. (A) Germination of *F. solani* conidia in *M. robertsii* cell free culture extract. *F. solani* conidia were suspended in different concentrations (100%, 90%, 50% and 10%) of 4 d old and 10 d old *M. robertsii* cell free culture filtrates. Germination of *F. solani* conidia was counted (N=4) after 24hr (light bars) and 48hr (dark bars). Each bar represents mean  $\pm$  S. E. ( $t$  test:  $P < 0.05$ ,  $n = 4$ , \*:  $P > 0.05$ , not statistically significant). (B) *F. solani* biomass yield in *M. robertsii* cell free culture filtrate (autoclaved – light bars and not autoclaved – dark bars) on day 4. Data represents the mean  $\pm$  S.E. of dry weights of *F. solani* produced in different concentrations (100%, 90% and 50%) of *M. robertsii* cell free extract and control.

After 4 weeks, roots were collected from all plant and checked for growth and disease indices. Roots obtained from *F. solani* treatment were necrotic when compared to roots obtained from controls as well as *F. solani* + *M. robertsii* treatment plants (Fig. 8B). Roots infected with *F. solani* were severely infected as compared to *F. solani* + *M. robertsii* treatment (Fig. 8B). Percent root rot area rating for *F. solani* treatment was greater ( $0.69 \pm 0.03$ ) than sterile controls ( $0.25 \pm 0.03$ ; *t* test:  $P < 0.0001$ ,  $t = 8.86$ ,  $df = 14$ ) and *F. solani* + *M. robertsii* treatments ( $0.34 \pm 0.03$ ; *t* test:  $P < 0.0001$ ,  $t = 8.37$ ,  $df = 14$ ). There were no statistical differences in root rot rating between the control and *F. solani* + *M. robertsii* treatment (*t* test:  $P > 0.05$ ,  $t = 1.97$ ,  $df = 14$ ). Additionally, the disease index based on intensity of hypocotyl and root rot was also higher in the *F. solani* treatment ( $3.93 \pm 0.07$ ) as compared to controls ( $1.73 \pm 0.28$ , *t* test:  $P < 0.0001$ ,  $t = 9.89$ ,  $df = 14$ ) and *F. solani* + *M. robertsii* ( $2.31 \pm 0.11$ , *t* test:  $P < 0.0001$ ,  $t = 12.22$ ,  $df = 14$ ).

*Metarhizium* has been previously shown to have antifungal activity against *Fusarium oxysporum*, *Botrytis cinerea*, and *Alternaria solani* (Kang et al., 1996). Other insect pathogenic fungi, including *Beauveria bassiana* and *Lecanicillium* spp., have also been shown to provide dual biological control properties (i.e. against both insect pests and plant pathogens) (Ownley et al., 2010). *M. anisopliae* and *B. bassiana* (ATCC18514) were reported to show in-vitro inhibition of *Ceratocystis ulmi* (Gemma et al., 1984). *B. bassiana*, which is also able to colonize endophytically, provided plant protection against *Rhizoctonia solani* and *Pythium myriotylum* (Ownley et al., 2008). The mechanism by which *Metarhizium* provides plant protection against plant fungal pathogens is not known, but results suggest that an extracellular, water soluble metabolite is implicated. It has been suggested that *M. anisopliae* inhibition of *Fusarium oxysporium* f. sp. *vasinfectum* could also be due to competition for nutrition and space and antibiosis (Qi et al., 2010). Multiple mechanisms could be involved. It has also been suggested

that *B. bassiana*, *Lecanicillium* spp. and *Trichoderma* spp. induce systemic resistance in plants (Owenly, 2010; Harman et al., 2004).

It has been previously shown that *M. robertsii* is a plant endophyte (see chapter 8), and here antagonism against a fungal plant root pathogen is shown. More complete understanding of fungal ecology is likely to aid in not only the development of more efficient insect and phytopathogen biocontrol programs, but also beneficial traits such as increased yields (Kabaluk and Ericsson, 2007), direct antagonism, compatibility with other beneficial microorganisms, plant growth and root system promotion (see chapter 8).

## 10. Conclusion

Studies of the interactions between plant roots and their associated microbiota have provided the basis for many insights in the areas of plant pathology and agronomy. These insights, in turn, have led to improvements in preventing plant diseases and increasing plant productivity. Furthermore, the unraveling of bacterial and fungal associations with plant roots has enhanced understanding in fundamental areas of biology and ecology, including symbiosis, gene transfer, coevolution, nitrogen fixation, energy flow, and nutrient cycling. In recent years, the possible roles of a biocontrol agent as plant growth promoter, plant endophyte and plant-pathogen antagonist may also provide an avenue for further understanding of fundamental biological processes.

In this study, very important aspects of *M. robertsii* with regard to rhizosphere, plant associations, plant root growth promotion and plant protection against a fungal pathogen have been uncovered. *M. robertsii* has never before been reported as a plant root endophyte. Being able to colonize plant roots enhances its capability as a biocontrol agent. *M. robertsii* presence helps in plant root development as well, which is again beneficial to plants. Also, *M. robertsii* inhibits bean root rot pathogen *Fusarium solani* f. sp. *phaseolis*, both in-vitro and in-vivo. Thus this study confirms *M. robertsii* not only as insect pathogenic fungus, but also as endophytic, plant root development promoter as well as biofungicide (Table 1).



**Table 1.** Comparison of *Metarhizium* with *Trichoderma* and *Piriformospora indica*.  
(Modified from: Shores *et al.*, Annu. Rev. Phytopathol,2010)

<b>Plant effect or mechanism</b>	<i>Metarhizium</i> spp.	<i>Trichoderma</i> spp.	<i>Piriformospora indica</i>	Plant growth promoting rhizobacteria
<b>Internal root colonization</b>	+	+	+	+
<b>Improved plant shoot and root growth</b>	+	+	+	+
<b>Enhanced root development</b>	+	+	+	+
<b>Increased plant nitrogen use efficiency</b>	?	+	+	/+
<b>Antagonist to phyto-pathogens</b>	+	+	/	+

This table has been adopted from Shores *et al.* Annu. Rev. Phytopathol, 2010; where authors were comparing *Trichoderma* with *P. indica* and plant growth promoting rhizobacteria. Since *Metarhizium* is also showing same properties, so I have added a column with *Metarhizium*. Here, += presence of effect or mechanism; ? = Effect or mechanism not confirmed; /= nothing known about the presence of effect or mechanism.

## 11. Future work

We now know that the insect-pathogenic fungus *Metarhizium robertsii* is capable of associating endophytically with plant roots (see chapter 8). This association has also proved to be beneficial for plants, as plants associated with *M. robertsii* show root growth promotion (see chapter 8). However, we still lack in our understanding of molecular mechanisms associated with plant root growth promotion in the presence of *M. robertsii*. To initiate such a study, effects of *M. robertsii* *in vivo* associations on some root hair related genes like the expansin family of root elongation genes in beans (Lee et al., 2003), and *KOJAK* family of root hair cell morphogenesis genes (Favery et al., 2001) could be done.

The efficacy of biological treatments for seed protection may largely depend upon the relative rates of colonization of seeds by antagonist and pathogen. It is therefore essential to somehow give the biocontrol agent a pre-start, without at the same time stimulating the pathogen. Therefore, strategies such as double coating seeds with the antagonist could be employed as an effective treatment against pathogen (Taylor *et al.*, 1991), as is currently being used for *Trichoderma* (Tronsmo and Hjeljord, 1998).

My study of antagonistic activity of *Metarhizium robertsii* on *Fusarium solani* f. sp. *phaseolis* is an initial recognition of the antagonistic effect of *M. robertsii* on *F. solani*. We now know that *M. robertsii* releases certain anti-fungal compounds against *F. solani*. However, analysis of enzymes released by *M. robertsii* in presence of plants and phytopathogen could also give valuable information. Certain enzymes like chitinase,  $\beta$ -1,3-glucanase, cellulase and PAL (Mohr et al., 1998; Mishra, 2010) are specifically linked to plant defence expression systems. Study on upregulation of such plant defence expression enzymes in presence of *M. robertsii* alone or with phytopathogen would ascertain whether *M. robertsii* is able to induce systemic

resistance in plants. Studies in chapter 9 also suggest that some of the anti-fungal compounds are heat stable. These compounds could be isolated and their effects on pathogens could also be monitored.

Not only plant systems, but specific fungal gene upregulations could also be studied. For example: the model system *Trichoderma*, secretes a proteinaceous elicitor Sm1 which further induces plant defence responses (Djonovic et al., 2006). While my study focus on antagonistic activity of *M. robertsii* against the host-specific phytopathogen *F. solani*, other phytopathogens like *Verticillium* and *Pythium* could also be used. Studying time course interactions between *Metarhizium* and plant pathogens while interacting would further give an insight into the mechanisms employed by *Metarhizium* to protect plants from fungal pathogens.

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## **APPENDIX I**

### **Selection of *M. robertsii* for plant association studies**

Three different species of *Metarhizium*: *M. robertsii*, *M. brunneum* and *M. guizhouense*, were tested for their ability to associate with plants. For this purpose, conidial suspensions ( $10^7$  conidia/ mL) of ARSEF 2575 (*M. robertsii*), 43a-2i (*M. brunneum*) and B77-ai (*M. guizhouense*) were inoculated (1% v/v) into switchgrass root exudate and were incubated at 15°C or 27°C. Conidial germination was assessed microscopically (Leitz DIAPLAN light microscope) at 12, 24 and 48 h. YPD inoculated with respective fungal conidia was used for positive controls.

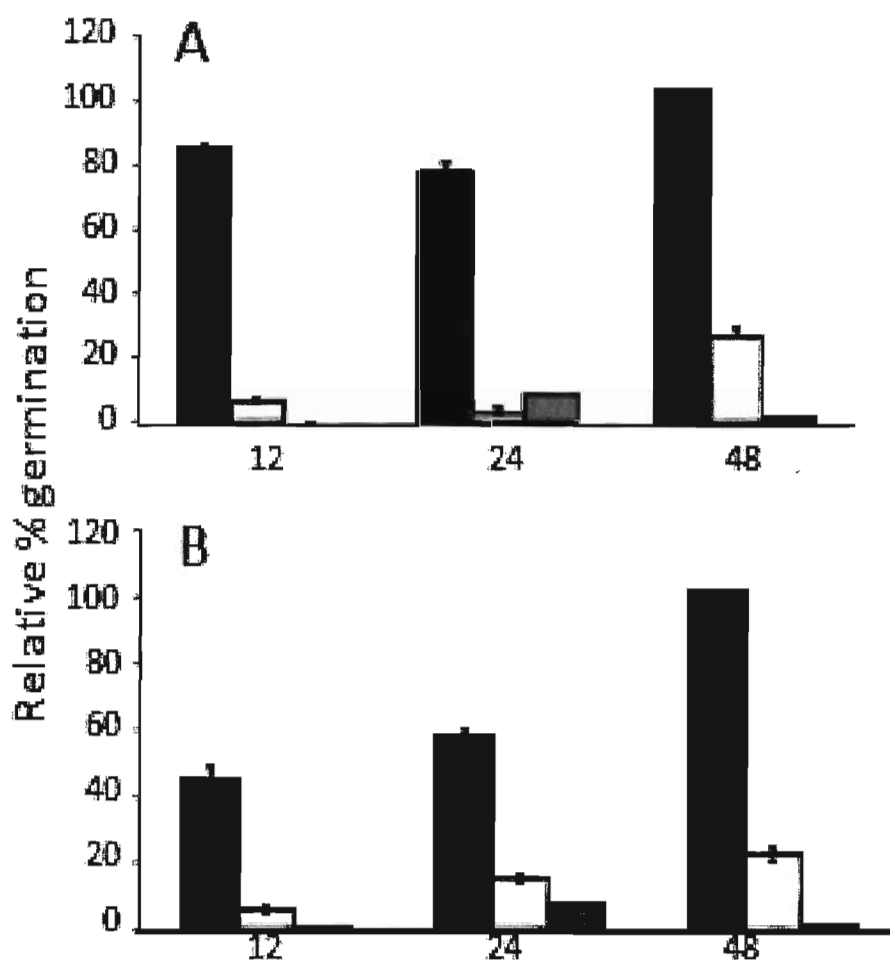
Switchgrass root exudate was collected from surface-sterilized seeds soaked in water in Petri dishes. Dishes were kept on an orbital shaker and provided with light throughout the period. Once 90% of the seeds had germinated, samples were kept on the shaker for an additional 4 days, after which root exudate was collected by vacuum filtration.

As another control, conidia from all three species of *Metarhizium* were assessed for germination in water. However, conidia failed to germinate in water for all three species. Conidia were considered as germinated when the germ tube was longer than the conidial width. Germination was scored for 100 conidia, and scoring was done three times for each species. The experiment was done twice.

## **RESULTS and CONCLUSION**

Three different species of *Metarhizium* showed differences in germination when grown in switchgrass root exudates (Fig 10). Results are expressed as germination rates obtained in switchgrass root exudates relative to those obtained in YPD, as a positive control. Among the three *Metarhizium* species tested, *M. robertsii* germinated well in switchgrass root exudates and had higher hyphal growth at 24h. *M. robertsii* also had higher conidia germination rate in root exudates than YPD at both 27°C and 15°C after 48h. However, *M. brunneum* and *M. guizhouense* showed minimal germination in switchgrass root exudate, relative to YPD. The differences in germination rates of *M. robertsii*, *M. brunneum* and *M. guizhouense* were statistically significant for all the observations obtained (t tests  $P \leq 0.0005$ ). These results indicate favourable association of *M. robertsii* with switchgrass root exudate. Whereas, *M. brunneum* and *M. guizhouense* were not able to germinate well in root exudate. Thus, depending on its

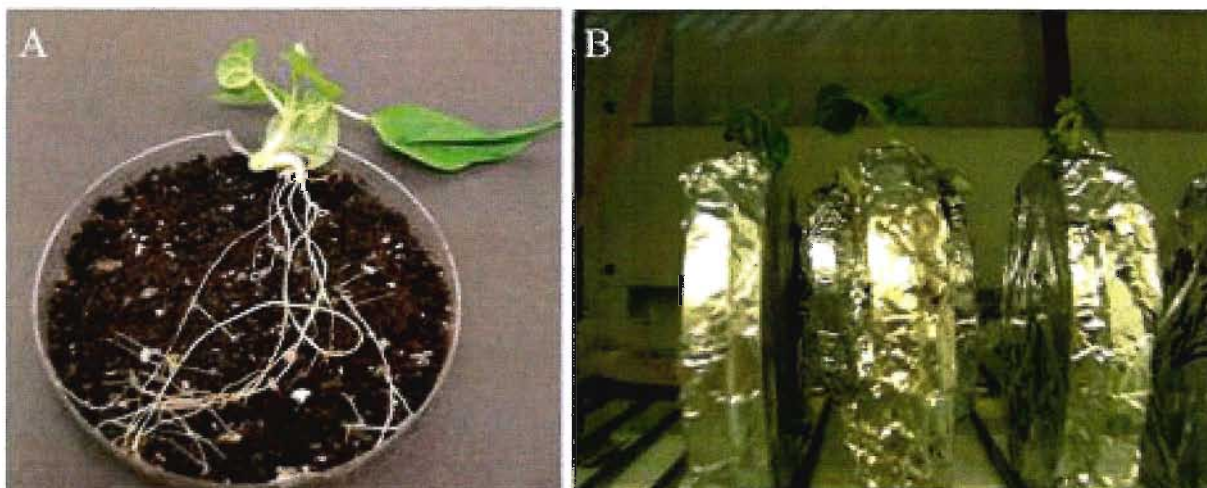
capability to germinate and grow well in root exudate, *M. robertsii* was used for further studies on associations with plants.



**Figure 10.** Relative germination rates of *M. robertsii* (black bars), *M. brunneum* (white bars) and *M. guizhouense* (grey bars) conidia inoculated into *Panicum virgatum* (switchgrass) root exudate at 27°C (a) and 15°C (b). Germination rates were relative to YPD as the positive control. Conidia failed to germinate in water for all three species. Germination was scored from 100 conidia; mean  $\pm$  SD are shown (n=3). The experiment was repeated twice with similar results.

## APPENDIX II

### Experimental setup for plant experiments in Chapter 8.



**Figure 11.**

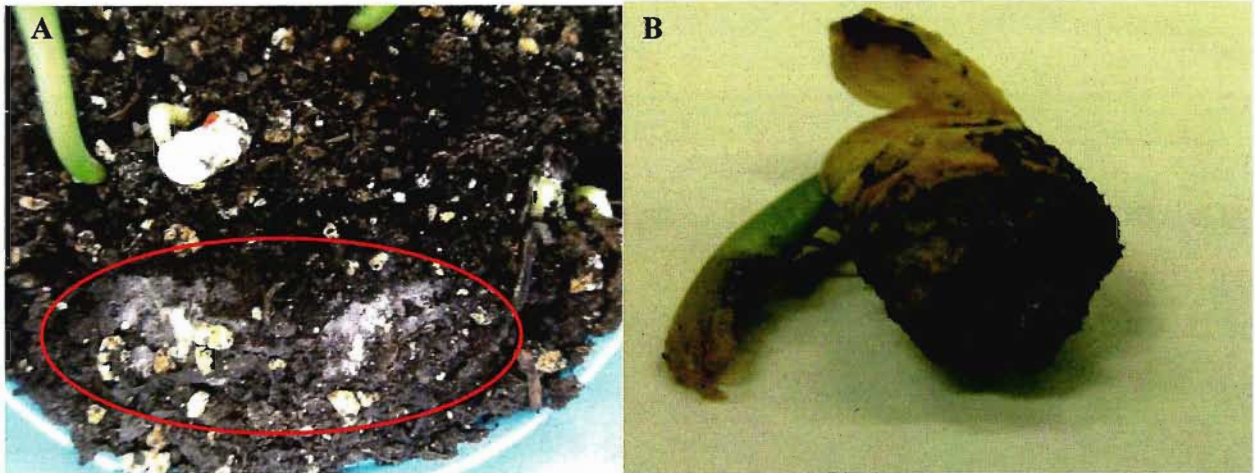
Bean seeds were surface sterilised and allowed to grown on moist filter papers till they develop into seedlings. These seedlings were then transferred to setup as shown in (A). The plants were then placed in growth chamber as shown in (B).

- A) Bean root as placed in Petri dish so that root is placed on soil inside the dish and shoot is left out of the dish.
- B) Vertically placed Petri dishes with bean seedlings.

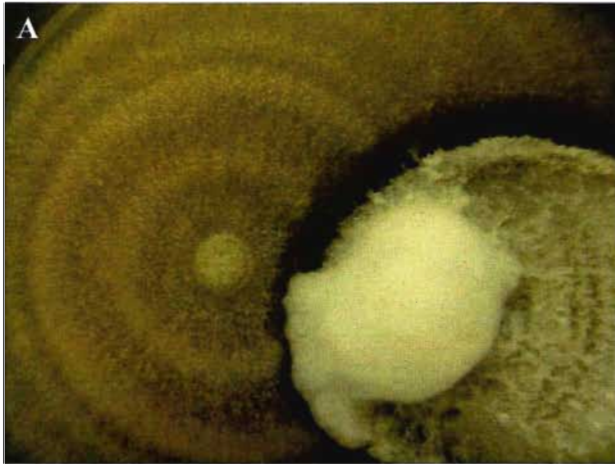
The roots from this setup were used for endophytic images.

### APPENDIX III

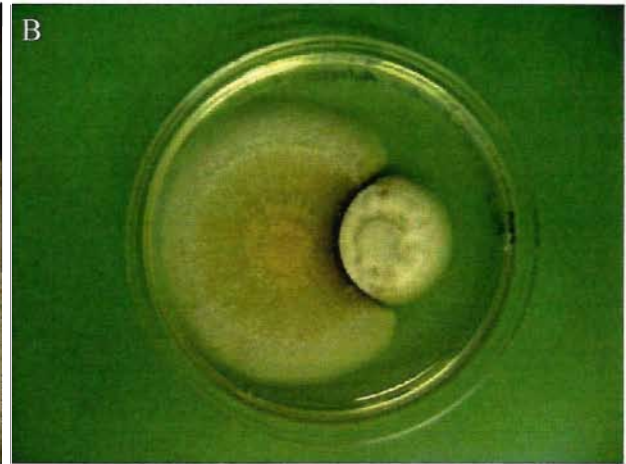
Images from *Metarhizium robertsii* antagonism to *Fusarium solani* f. sp. *phaseolis*.



**Figure 12.** Images from plant experiments: (A) Fungal growth on soil surface on day 11 of bean seed emergence. Treatment with only *F. solani* showed necrosis in bean seeds before they could germinate. Pathogenic fungus (*F. solani* f. sp. *phaseolis*) could then be seen as a white mycelial mass on the soil surface. (B) Bean seedling from a *F. solani* treatment after 4 weeks, damping off could be seen.



*F. solani* / *M. robertsii*



*F. solani* / *M. robertsii*

**Figure 13.** *M. robertsii* - *F. solani* interaction in dual cultures. (A) *M. robertsii* shows increased mycelial growth at the interface with *F. solani*. Zones of inhibition are observed by *M. robertsii* on *F. solani* growth. (B) The zone of inhibition of *F. solani* by *M. robertsii*.

## APPENDIX IV

### Statistical significance data analysis

**Table 2.** Statistical significance (t-test,  $\alpha = 0.05$ ) for *F. solani* conidia germination in different concentrations (100%, 90%, 50% and 10% v/v) of *M. robertsii* cell free extract (obtained on day 4 and day 10) and control (Water yeast extract broth) at 24 h. and 48h.

	100% 4D- 24h.	100% 4D- 48h.	90% 4D-24h.	90% 4D-48h.	50% 4D- 24h.	50% 4D- 48h.	10% 4D- 24h.	10% 4D- 48h.	Control 24h.
#1	27	44	28	40	33	48	35	66	62
#2	26	44	30	45	28	49	38	67	68
#3	21	39	28	47	32	47	34	63	71
#4	19	43	27	42	35	43	37	61	68
Mean	23.25	42.5	28.25	43.5	32	46.75	36	64.25	67.25
S.D.	3.8622	2.3805	1.2583	3.1091	2.9439	2.6599	1.8257	2.7538	3.7749
S.E.	1.9311	1.1902	0.6292	1.5546	1.4720	1.3149	0.9129	1.3769	1.8875
	100% 10D- 24h.	100% 10D- 48h.	90% 10D- 24h.	90% 10D- 48h.	50% 10D- 24h.	50% 10D- 48h.	10% 10D- 24h.	10% 10D- 48h.	Control 48h.
#1	11	21	16	29	20	38	24	40	63
#2	11	26	16	30	21	36	25	38	73
#3	14	24	18	30	15	31	25	34	70
#4	10	24	16	27	21	32	30	37	74
Mean	11.5	23.75	16.5	29	19.25	34.25	26	37.25	70
S.D.	1.7321	2.0616	1.0	1.1412	2.8723	3.3040	2.7080	2.5	4.9666
S.E.	0.8660	1.0308	0.5	1.5546	1.4361	1.6520	1.3540	1.25	2.4833
t-stat	5.3104	11.9083	14.6210	8.4903	6.1999	5.9200	6.1237	14.5188	0.8816
P value	0.0130	<0.0001	<0.0001	<0.0001	0.0008	0.0010	0.0009	<0.009	*0.4119

\*=  $P > 0.05$ , thus data are statistically not significant. The number of *F. solani* conidia germinated/ 100 cells.



**Table 3.** Statistical significance (t-test,  $\alpha = 0.05$ ) for *F. solani* conidia germination in different concentrations (100%, 90%, 50% and 10%) of *M. robertsii* cell free extract obtained on day 4 and day 10 compared to control (Water yeast extract broth) at 24h and 48 h.

	<b>24h.</b>				<b>48h.</b>			
	<b>100%</b>	<b>90%</b>	<b>50%</b>	<b>10%</b>	<b>100%</b>	<b>90%</b>	<b>50%</b>	<b>10%</b>
<b>4D</b>								
t-stat	16.2944	19.6024	14.7270	14.9049	9.9863	9.0452	8.2742	2.0250
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	0.0002	*0.0893
<b>10D</b>								
t-stat	26.8460	25.9915	20.2386	17.7580	17.2016	15.8792	11.9862	11.7800
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.009

\*= P value > 0.05, thus data are not statistically significant with respect to control.

4D: *M. robertsii* cell free culture filtrate obtained after 4 days of *M. robertsii* growth.

10D: *M. robertsii* cell free culture filtrate obtained after 10days of *M. robertsii* growth.

**Table 4.** Statistical significance (t-test,  $\alpha=0.5$ ) for disease indices in roots obtained from control, *F. solani* (diseased) and *F. solani* + *M. robertsii* (treated) plants. Plants were harvested 4 weeks after respective treatments and planting in soil. Disease index was observed as percent root rot area referred as scale 1. Disease index rating is: 0 - no root rot symptoms; 0.10 – less than 10% root area rotted; 0.25 – 11-25% root area rotted; 0.50 – 26-50% root area rotted; 0.75 – 51-75% root area rotted; 1.00 – more than 75% root area rotted.

	<b>Control</b>	<b><i>F. solani</i> (diseased)</b>	<b><i>F. solani</i> + <i>M. robertsii</i> (treated)</b>
Mean	0.2533	0.6786	0.3438
S.D.	0.1172	0.1172	0.125
S.E.	0.0303	0.0313	0.0313
	<b><i>F. solani</i> (diseased)</b>	<b><i>F. solani</i> + <i>M.</i> <i>robertsii</i> (Treated)</b>	<b>Control</b>
Mean	0.6786	0.3438	0.2533
S.D.	0.1172	0.125	0.1172
S.E.	0.0313	0.0313	0.0303
t-stat	8.8657	8.3666	1.9765
P value	<0.0001	<0.0001	*0.0681

\*= P value >0.05, thus data are not statistically significant with respect to each other.

**Table 5.** Statistical significance (t-test,  $\alpha=0.5$ ) for disease indices in roots obtained from control, *F. solani* (diseased) and *F. solani* + *M. robertsii* (treated) plants. Plants were harvested 4 weeks after respective treatments and planting in soil. Disease index was observed as intensity of hypocotyl rot and root rot referred as scale 2. Disease index rating is: 0 - no root rot; 1 - mild hypocotyl browning, roots firm; 2 – hypocotyl browning, but roots falling off on pressure; 3 – severely damaged hypocotyl and roots very fragile and coming off easily; 4 – damping off after seed germination; 5 – damping off occurred in seed.

	<b>Control</b>	<b><i>F. solani</i> (diseased)</b>	<b><i>F. solani</i> + <i>M. robertsii</i> (treated)</b>
Mean	1.7333	3.9286	2.3125
S.D.	1.0712	0.2673	0.4787
S.E.	0.2768	0.0715	0.1197
	<b><i>F. solani</i> (diseased)</b>	<b><i>F.solani</i> + <i>M.</i> <i>robertsii</i> (Treated)</b>	<b>Control</b>
Mean	3.9286	2.3125	1.7333
S.D.	0.2673	0.4787	1.0712
S.E.	0.0715	0.1197	0.2768
t-stat	9.8859	12.2202	2.3577
P value	<0.0001	<0.0001	0.0335

Since P value <0.05, data are statistically significant for all treatments.

**Table 6.** Statistical significance (t-test,  $\alpha=0.05$ ) of *F. solani* biomass yield in *M. robertsii* cell free culture filtrate (autoclaved and not-autoclaved) on day 4. *F. solani* was allowed to grow for 4 days in different concentrations (100%, 90% and 50%) of *M. robertsii* autoclaved and not-autoclaved cell free extract. The culture was filtered and dry weight of *F. solani* was measured.

	4D			10D		
	100%	90%	50%	100%	90%	50%
<b>Autoclaved <i>M. robertsii</i> cell free extract</b>						
Mean	0.0585	0.381	0.457	0.029	0.214	0.301
S.D.	0.0170	0.0376	0.0598	0.0099	0.0647	0.0461
S.E.	0.0054	0.0119	0.0189	0.0031	0.0205	0.0146
<b>Not- autoclaved <i>M. robertsii</i> cell free extract</b>						
Mean	0.045	0.394	0.4	0.022	0.231	0.252
S.D.	0.0196	0.0467	0.0715	0.0103	0.0468	0.0266
S.E.	0.0061	0.0148	0.0226	0.0033	0.0148	0.0084
t-stat	1.7285	0.73	2.0053	1.5800	0.6963	3.0389
P value	0.1010	0.4747	0.0602	0.1315	0.4951	*0.0071

\*=  $P < 0.05$ , thus data are statistically significant.

For all the data analysis done it was assumed that samples were obtained from populations with normal distribution.

S.D. Standard deviation  
S.E. Standard error of the Mean  
t-stat Student t-test statistic  
P value The probability. It is the smallest level of significance for which the observed sample statistic rejects the null hypothesis for the performed two-tailed t-test with a 5% level of significance ( $\alpha = 0.05$ ).